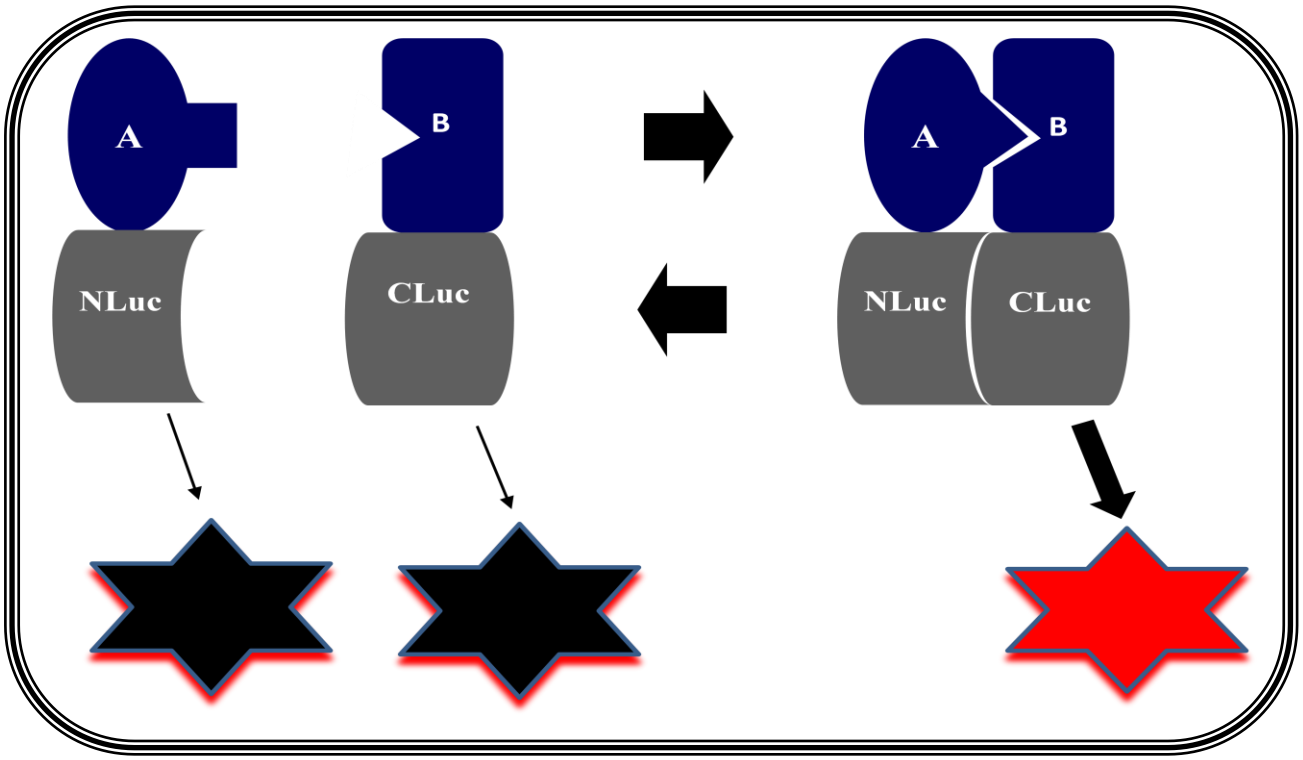
Protein-protein interaction assay with split luciferase *in planta*



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Protein-protein interactions (PPIs) regulate many different cellular processes including transcription, translation, cell division, signal transduction, and oncogenic transformation. It is therefore important to develop sensitive and versatile techniques for the detection of these protein-protein interactions in order to fully understand protein functions. The most commonly used and traditional technique, the yeast two-/three hybrid (Y2H/Y3H) method, often results in false positives and false negatives, and other widely used techniques, such as bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET), and bimolecular fluorescence complementation (BiFC) require extensive instrumentation. When compared with other PPI detection methods, the luciferase-based complementation assay specially split luciferase is believed to deliver the most sensitive and highest dynamic range, making it ideal for large-scale analysis. Therefore, for testing PPIs *in planta*, split *Renilla* luciferase complementation assay was chosen. In order to conduct this experiment, a series of plasmid constructs were made to enable the transient expression of fusion proteins. A well known protein pair, *Arabidopsis* nuclear Histone 2A and 2B, was tested initially as a proof of concept, and then three more proteins of the *Gerbera* MADS-box B class were investigated. For *Arabidopsis* Histone 2A and 2B, the intensity in all combinations was on average 9.4-fold higher in Relative Luminescence Units (RLUs) than the mock treated protoplasts. Moreover, in the case of *Gerbera* MADS-box B class proteins, the protein pairs GDEF1-GDEF2, GDEF1-GGLO1, and GDEF2-GGLO1 showed 8.4-19.4, 9.5-15.8, and 8.3-9.1-fold higher signals than the mock treated protoplasts. These results suggest that various complexes formed from different combinations of these three B class MADS-box proteins may increase the complexity of their regulatory functions, thus specifying the molecular basis of whorl morphogenesis and combinatorial interactions of floral organ identity genes in *Gerbera*. Finally, it was concluded that split *Renilla* luciferase can be a simple, reliable, fast, and effective method for examining PPIs *in planta*.

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Protein-protein interaction, split *Renilla* luciferase, MADS-box genes, flower development, vector construction, polyethylene glycol, electroporation, luminometer.

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Glossary

Chromophore: the chemical group that gives color to a molecule.

Clade: a group of biological taxa or species that share features inherited from a common ancestor.

Fluorophore: molecule or functional group which is capable of fluorescence.

Heterodimer: a dimer or complex of two different molecules, usually proteins.

Heterologous: means derived from organisms of a different but related species.

Homeotic: a mutation that causes one member of a repetitive series to assume the identity of another member. For example, the transformation of sepals into petals.

Homodimer: a dimer or complex of two of the same molecule, usually a protein.

Interactome: the whole set of molecular interactions in cells.

Orthologue: groups of homologous genes (means have evolved from a common ancestral gene) which are split by speciation and have the same function in two species.

Paralog: groups of homologous genes which are duplicated inside a particular species and can evolve new functions from the original one.

Phylogenetic: the study of evolutionary relatedness among various groups of organisms (for example: species, populations etc.), which is discovered through molecular sequencing data and morphological data matrices.

Signal transduction: it is a biological process by which extracellular signals are moved to the inside of the cell.

Whorl: organs of the same structure and function that are arranged in a concentric ring. In the flower, the outermost whorl (whorl 1) develops first and contains the sepals, followed by the petals, stamens and carpels in whorls 2, 3 and 4 respectively.

Zygomorphic: a flower capable of being cut in only one plane so that the two halves are mirror images.

Abbreviations

×g	Gravitational force
35S:	Cauliflower mosaic virus 35S promoter
6xHis:	Peptide tag encoding His-His-His-His-His-His
ATP:	Adenosine triphosphate
attL1, L2:	Lambda recombination site L1 or L2
attR1, R2:	Lambda recombination site R1 or R2
BAP:	Benzylaminopurine
BSA:	Bovine serum albumin
Cre-loxP:	Cre is the enzyme; loxP is the bacteriophage P1 recombination site
cv.:	Cultivar
DDW:	Distilled deionized water
DTT:	Dithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
Eqv.:	Equivalent
FlAsH:	Fluorescence arsenical helix
LB:	Luria-Bertani
MES	2-(*N*-morpholino) ethanesulfonic acid
NAA:	Napthalene acetic acid
OD:	Optical density
pp:	Protoplast
PET:	Positron emission tomography
PVP:	Polyvinylpyrrolidone
RCF:	Relative centrifugal force
RPM:	Revolutions per minute
RT:	Room temperature
SPECT:	Single photon emission computed tomography
TD:	Tracking dye (Bromophenol blue)
TetraCys:	Peptide tag encoding Cys-Cys-Pro-Gly-Cys-Cys
(x):	Times

Abstract

Protein-protein interactions (PPIs) regulate many different cellular processes including transcription, translation, cell division, signal transduction, and oncogenic transformation. It is therefore important to develop sensitive and versatile techniques for the detection of these protein-protein interactions in order to fully understand protein functions. The most commonly used and traditional technique, the yeast two-/three hybrid (Y2H/Y3H) method, often results in false positives and false negatives, and other widely used techniques, such as bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET), and bimolecular fluorescence complementation (BiFC) require extensive instrumentation. When compared with other PPI detection methods, the luciferase-based complementation assay specially split luciferase is believed to deliver the most sensitive and highest dynamic range, making it ideal for large-scale analysis. Therefore, for testing PPIs *in planta,* split *Renilla* luciferase complementation assay was chosen. In order to conduct this experiment, a series of plasmid constructs were made to enable the transient expression of fusion proteins. A well known protein pair, *Arabidopsis* nuclear Histone 2A and 2B, was tested initially as a proof of concept, and then three more proteins of the *Gerbera* MADS-box B class were investigated. For *Arabidopsis* Histone 2A and 2B, the intensity in all combinations was on average 9.4-fold higher in Relative Luminescence Units (RLUs) than the mock treated protoplasts. Moreover, in the case of *Gerbera* MADS-box B class proteins, the protein pairs GDEF1-GDEF2, GDEF1-GGLO1, and GDEF2-GGLO1 showed 8.4-19.4, 9.5-15.8, and 8.3-9.1-fold higher signals than the mock treated protoplasts. These results suggest that various complexes formed from different combinations of these three B class MADS-box proteins may increase the complexity of their regulatory functions, thus specifying the molecular basis of whorl morphogenesis and combinatorial interactions of floral organ identity genes in *Gerbera*. Finally, it was concluded that split *Renilla* luciferase can be a simple, reliable, fast, and effective method for examining PPIs *in planta*.

1. Introduction

1.1 Protein-protein interactions

1.1.1 A brief synopsis about protein-protein interactions

Protein-protein interactions (PPIs) are known to play key roles in the structural and functional organization of living cells because of their regulatory roles in processes such as transcription, translation, cell division, signal transduction, and oncogenic transformation. Many unsolved problems currently under investigation in the fields of molecular biology and biochemistry are related to PPIs. For example, signal transduction occurs by the process of PPIs and has a very essential role in a number of biological processes (such as metabolism, cellular proliferation, gene activation etc.) as well as in many diseases (like cancer, diabetes, heart disease, autoimmune disease etc.). A Protein may interact with another protein for modifying its DNA binding site, as in the case of HBV X protein which alters the DNA binding specificity of CREB and ATF-2 (Maguire *et al*., 1991). Hence, one of the central goals of current research in different biological fields is to identify these interactions and to characterize their physiological significance (Ladant and Karimova, 2000; Ozawa and Umezawa, 2001).

1.1.2 Different methods used to detect protein-protein interactions

Currently many methods are used to detect protein-protein interactions in living organisms but among them the traditional yeast two-three hybrid (Y2H/Y3H) method is still the most popular for many reasons, for example its ease of use and large-scale screening suitability. The main disadvantage of this heterologous method is the generation of both false positives and false negatives. To overcome these limitations, many reporter-based protein-protein interaction assays were later developed for measuring PPIs *in vivo* (**Table 1**). These techniques include fluorescence resonance energy transfer (FRET) (Ha *et al*., 1996; Mahajan *et al*., 1998; Pollok and Heim, 1999), bioluminescence resonance energy transfer (BRET) (Xu *et al*., 1999; Bertrand *et al*., 2002; Subramanian *et al*., 2006), and bimolecular fluorescence complementation (BiFC) (Hu *et al*., 2002; Hu and Kerppola, 2003) assays. Alternative reporter-based methods (such as *β*-galactosidase, 1-*β*-lactamase, murine dihydrofolate reductase etc.) were later developed using the

principle of protein fragment complementation joined with enzymatic assays to successfully detect PPIs in *Escherichia coli*, mammalian cells and *in planta* (Chen *et al.*, 2008).

Table 1: Strategies for detecting protein-protein interactions and their applications to various biological systems (adapted from Villalobos *et al*., 2007).

Strategy	**Lysates**	**Bacterial cells**	**Mammalian cells**	**Plant cells**	**Readout**	**Assay type**
Co-immunoprecipitation	+	-	-	-	Immunoblotting, fluorescence	Biochemical
Two hybrid	-	+	+	+	Fluorescence, bioluminescence, PET, SPECT	Transcriptional
Split ubiquitin	-	-	+	+	Fluorescence, bioluminescence, PET, SPECT	Transcriptional
FRET	+	+	+	+	Fluorescence	Posttranslational
BRET	+	+	+	+	Bioluminescence	Posttranslational
Split DHFR	-	-	+	+	Survival assay, fluorescence	Posttranslational
Split beta-lactamase	+	+	+	-	Fluorescence, absorbance	Posttranslational
Split beta-galactosidase	+	+	+	-	Fluorescence, absorbance	Posttranslational
Split fluorescent proteins	+	+	+	+	Fluorescence	Posttranslational
Split luciferases	+	+	+	+	Bioluminescence	Posttranslational
Split luciferase inteins	+	+	+	+	Bioluminescence	Posttranslational

1.1.3 PPI assay methods generally used *in planta*

The Y2H/Y3H assay is the most commonly used technique to detect PPIs in eukaryotic cells. It allows identification of gene encoding proteins that interact with target proteins. Its working principle is based on some eukaryotic transcription factors that are characterized by two separable functional domains (DNA binding and activation domains). One investigating protein is fused with the DNA binding domain and another one with the activation domain. If two proteins interact/bind then both transcription factors become connected indirectly with each other, which ultimately initiates transcription and, as a result, reporter gene expression occurs. The two-hybrid system utilizes the yeast GAL4 transcriptional activator protein, which is required for expression of gene encoding proteins involved in galactose metabolism. This

method is ideal for the large-scale screening of interacting proteins and the construction of protein interactomes (Fields and Song, 1989; Li *et al*., 2004). Other methods include the previously mentioned FRET assay, which in principle is an energy transfer that occurs when the fluorescent donor, Eu-chelate, and the fluorescent acceptor, allophycocyanin, come in close proximity to each other. If the titrated ligand modulates the interaction between the nuclear receptor and the peptide, the fluorescent signal is modulated in a ligand dose dependent manner. It enables the detection and sub-cellular localization of PPIs in living cells by monitoring energy transfer between donor and acceptor fluorophores that are fused to interacting proteins (Bhat *et al*., 2006). Another emerging technique is BRET, which uses a bioluminescent luciferase (generally *Renilla* luciferase) genetically fused to one experimental protein and a green fluorescent protein mutant (generally yellow fluorescent protein) fused to another protein of interest. Interactions between the two fusion proteins bring the luciferase and the green fluorescent protein closer distance for resonance energy transferring between them which ultimately change the color of the bioluminescent emission. This process allows for the detection of PPIs in live cells in real time, thus providing a new window into cellular signal transduction processes (Subramanian *et al*., 2004). The determination of PPIs in plants using BiFC is another process where the yellow fluorescent protein (YFP) is split into two non-overlapping N and C-terminal fragments. Each fragment is fused with a gene of interest, which enables the expression of fusion proteins. Reconstruction of YFP chromophore occurs only when the experimental proteins of interest interact with each other (Bracha-Drori *et al*., 2004; Walter *et al*., 2004). Other *in vivo* detection and imaging methods rely on the complementation of active enzymes from mutated proteins or protein fragments. These include complementation assays that are based on the reconstitution of murine DHFR, firefly, or *Renilla* luciferase (Remy and Michnick, 1999; Chen *et al*., 2008; Fujikawa and Kato, 2007).

1.1.4 Overview of the split luciferase system

In the split luciferase technique, one experimental protein is fused with the amino-terminal half of a reporter protein/enzyme and the other with the carboxyl-terminal half of the same reporter protein/enzyme. Now only physical interaction between the two experimental proteins reconstructs the two split halves of the reporter protein/enzyme which ultimately activates the enzymatic activities and can easily be measured by various *in vivo*/*in vitro* assays (**Figure 1**).

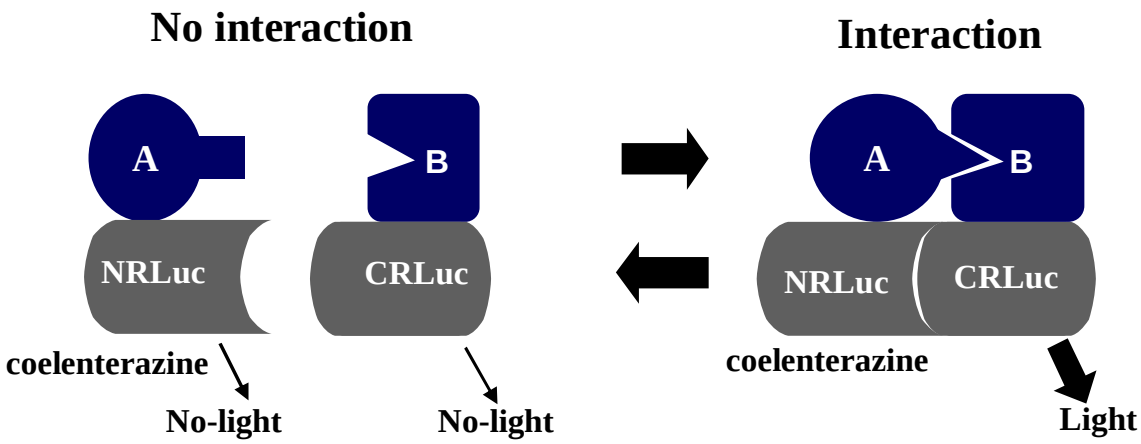

Figure 1: Split *Renilla* luciferase method (adapted from Fujikawa and Kato, 2007).

Proteins of interest are shown as A and B. Bold arrows show the dynamics of the protein-protein interaction. NRLuc (N-terminal domain of *Renilla* luciferase), CRLuc (C-terminal domain of *Renilla* luciferase) and coelenterazine (substrate of *Renilla* luciferase).

1.1.5 Advantages of using split *Renilla* luciferase

The first major advantage of this method is that there is no need for an expensive and sophisticated microscope to detect the luminescence signal. However, to maximize the advantage of using split *Renilla* luciferase over other well established techniques (e.g., Y2H/Y3H, FRET, BRET, and BiFC) a number of prerequisites needed to be met: (i) high sample number, (ii) the PPI is conducted by an outside signal and the time duration is a longer period, and (iii) already known sub-cellular localization of the interacting proteins (Fujikawa and Kato, 2007). Fujikawa and Kato (2007) also reported that split luciferase can measure protein dissociation but this feature is absent in BiFC. *Renilla* luciferase also has a lot of desirable features like its small size (36 kDa), very strong luminescence, and its ATP independence (Ozawa, 2008). Ozawa (2008) also observed that *Renilla* luciferase substrate coelenterate luciferin (coelenterazine) is able to penetrate rapidly through cell membranes thus it allows live cell assays and *in vivo* imaging. This split *Renilla* luciferase reporter assay facilitates accurate and sensitive detection of PPIs and it's possible to evaluate quantitatively in a high-throughput manner (Kim *et al*., 2004). Luker and Piwnica-Worms (2004) stated that the sensitivity and dynamic range of split luciferase is higher due to its optimized substrate (high cell permeability and high quantum yield), making it ideal for large-scale analysis.

1.2 *Gerbera* as a model plant

1.2.1 *Gerbera hybrida* as a model for flower development

Plant genetics, including flower development biology, is primarily studied using *Arabidopsis thaliana* of the Brassicaceae family which has transformed this valueless weed variety into one of the most important model organisms (Meyerowitz and Somerville, 1994). But the traditional model plants for studying floral organ development (like *Arabidopsis*, *Antirrhinum*, *Petunia* etc.) lack some important features that are present in *Gerbera*, a member of the sunflower family (Teeri *et al*., 2006). *Gerbera hybrida* is a typical species in the family Asteraceae and its inflorescences are composed of morphologically different types of flowers that are firmly packed into a single flower head that visibly represent a single flower (Harris, 1995). For example, marginally located ray flowers of *Gerbera* are all female and strongly zygomorphic as well as in the pseudanthium there are two to three cycles of showy petal-like structures present (Yu *et al*., 1999). Then centrally located disc flowers are hermaphrodite and their corolla whorls are much less prominent. In between ray and disc flowers, *Gerbera* has a third flower type called trans which are all female like ray flowers but their corolla whorls are similar to that of the disc flowers. But not all Asteraceae family flowers show similar features like *Gerbera*. For example, the sunflower has hermaphrodite central flowers and sexually neutral ray flowers, while *Gerbera* ray flowers are female (Bremer, 1994). *Gerbera* shows different type of flower formation within their inflorescences but this special feature is absent in common model plants like *Arabidopsis* and *Antirrhinum*. This feature may be an essential factor in the evolutionary success of this group of flowering plants and can also help in the study of higher-order developmental regulation genetics by using single *Gerbera* genotypes (Teeri *et al*., 2006).

1.2.2 The MADS-box transcription factors

The MADS-box genes are found in a eukaryotic family of transcription factors called the MADS-box protein family and their sequences are highly conserved (Shore and Sharrocks, 2005). The name MADS box was constructed from the first four members of the family MCM1, AGAMOUS, DEFICIENS, and SRF (serum response factor) of yeast, *Arabidopsis*, *Antirrhinum*, and humans respectively (Saedler and Huijser, 1993). Typically, higher order eukaryotes, like humans, and some other lower order eukaryotes (e.g., *Drosophila melanogaster*, *Saccharomyces*

cerevisiae, and *Caenorhabditis elegans*) genomes contain only twenty, six, twenty two, and three MADS-box genes, respectively (http://www.ebi.ac.uk/interpro/DisplayIproEntry?ac=IPR002100). But more than one hundred of these genes are normally found (e.g., 107 in *Arabidopsis thaliana*) in the case of flowering plants (Pařenicová *et al*., 2003; Teeri *et al*., 2006). Plant MADS-box genes encode proteins that share a stereotypical MIKC structure with the highly conserved DNA-binding site of it (Alvarez-Buylla *et al*., 2000a, b). Plant genomes contain another moderately conserved domain called K-box which is found in the central portion of these proteins, a unique subgroup of MADS-box genes, important for PPIs and form a coiled-coil structure (Teeri *et al*., 2006; Alvarez-Buylla *et al*., 2000a, b). In animals and fungi, two distinct types of MADS-box genes have been identified called the SRF and MEF2-like domains which are named as Type I and Type II MADS box genes, respectively, in the case of plants (Alvarez-Buylla *et al*., 2000a, b). On the other hand, K domain is found only in the Type II MADS domain sequences of plants which indicate that this domain evolved after this lineage diverged from the Type I MADS domain (Alvarez-Buylla *et al*., 2000b).

1.2.3 Roles of *Gerbera* MADS-box domain proteins

Flower development and floral induction in flowering plants are controlled by plant MADS-box genes. According to classical ABC model it was predicted that MADS domain proteins act in a combinatorial manner to define formation of sepals, petals, stamens, and carpels of floral organ determination (Coen and Meyerowitz , 1991; Bowman *et al*., 1991). Afterwards, the ABC model was further modified to include D and E functions (Angenent and Colombo, 1996; Theiβen, 2001). *Arabidopsis* and *Antirrhinum* were initially used as model plants for studying regulation of flower organ identity and it was found that a plant-specific clade of the eukaryotic-wide family of MADS-box regulatory factors are responsible (Coen and Meyerowitz, 1991). An important observation in floral homeotic mutations was that they affected successive pairs of the concentric whorls of flower organs. In the wild type angiosperm flower, whorl 1 (the outermost whorl) is engaged by sepals, whorl 2 by petals, whorl 3 by stamens, and whorl 4 by carpels. In A class homeotic floral mutants, organs in whorl 1 and whorl 2 develop into carpels and stamens, respectively. In B class mutants, sepals and carpels develop in place of petals and stamens in whorl 2 and whorl 3. Finally, the C class mutants show petals in whorl 3, sepals in whorl 4, and

this kind of structural sepals-petals-petals repetitions never ends until the floral meristem stops proliferating (**Figure 2**). Thus, in C class mutants, the determinacy of the flower is lost due to homeotic conversion (Coen and Meyerowitz, 1991).

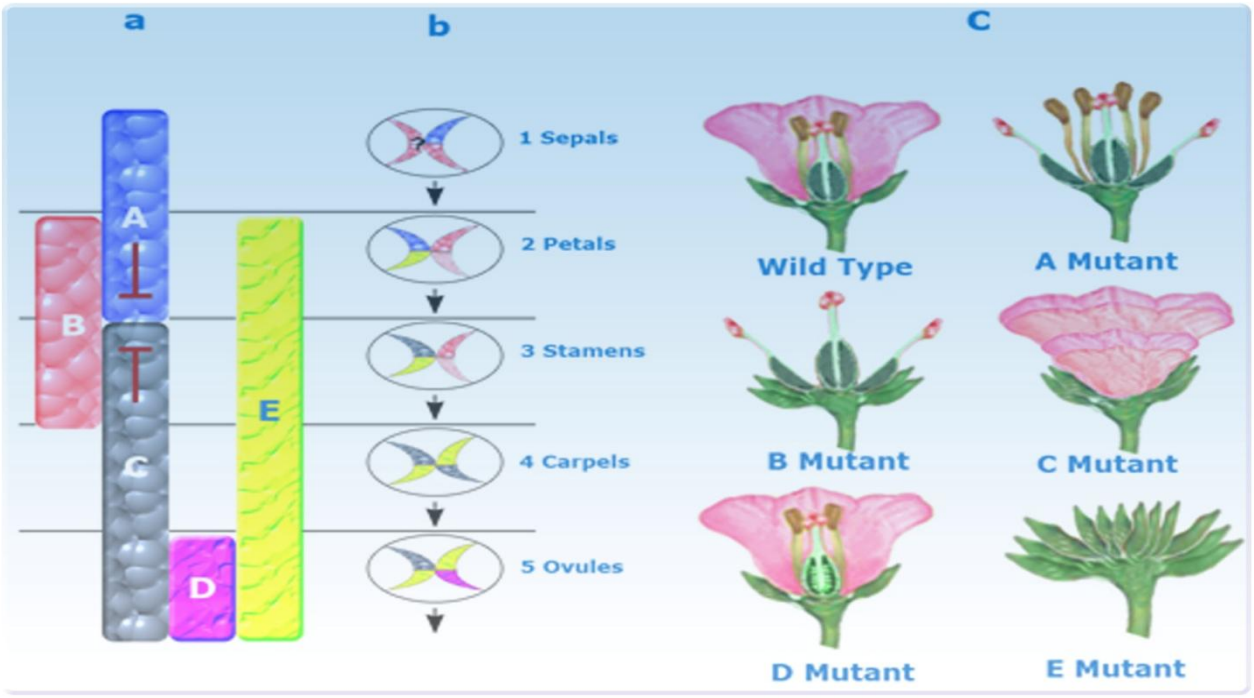

Figure 2: The floral 'ABCDE' model.

(a) In the 'ABCDE' model, the letters A, B, C, D, and E stand for the overhanging activities of five classes of regulatory genes that are responsible for the five floral organs identity: A and E class genes are needed for making the sepals; A, B, and E for the petals; B, C, and E for the stamens; C and E for the carpels; and finally C, D, and E for the ovules. (b) A schematic diagram of the assumed quaternary complexes bound to a target DNA sequence. The colors of the various proteins shown within the complexes resemble their functions in the 'ABCDE' model. The different B class proteins form heterodimers among themselves which are indicated by different shades of pink. (c) The theoretical composition of the five 'ABCDE mutants'. In the A mutant, the C function dominates over the A function in whorls 1 and 2; hence carpels are produced in whorl 1 and stamens in whorl 2. In the B mutant, sepals are produced in whorl 1 and whorl 2, and carpels in whorl 3 and whorl 4. In the C mutant, unlike the A mutant, the A function is active in all floral whorls. Therefore, petals are formed in both whorls 2 and 3 and the flower becomes indeterminate resulting in an iteration of the floral program and the production of a new floral bud from the center of the flower. In the D mutant, the ovules in the whorl 5 are converted into carpeloid organs. Finally, in the E mutant only sepals are produced in all five whorls and the flowers again become indeterminate and form a new floral bud from the central meristematic region.

The MADS-box genes, or the nucleotides that encode the MADS domain with its DNA-binding properties, is a 168 base-pair long sequence with absolute conservation concerning length and very high conservation concerning sequence (Alvarez-Buylla *et al*., 2000a, b). For that reason, isolation and identification of MADS-box genes in any given plant species is very

straightforward. Isolation and identification can be accomplished using a variety of techniques like screening of *Gerbera* cDNA libraries with heterologous probes, RT-PCR with MADS-box specific primers etc. Most recently, mining of the *Gerbera* EST collection has isolated twenty *Gerbera* cDNAs encoding MADS- box domain proteins (Yu *et al*., 1999; Kotilainen *et al*., 2000; Laitinen *et al*., 2005, Satu Ruokolainen, unpublished).

Candidates representing all three ABC floral model functions are provided by the phylogenetic analysis of *Gerbera* MADS-box genes (Yu *et al*., 1999). *Gerbera* A class gene *GSQUA1* does not behave like an A function gene in *Arabidopsis*, although it is evidently a member of the A function clade according to phylogenetic analysis. *AP1* in *Arabidopsis* and *SQUA* in *Antirrhinum* both are expressed in Whorls 1 and 2, but *SQUA1* in *Gerbera* is not. It is flower abundant, but expressed in the vascular tissue of the ovaries rather than in floral organ primordia (Yu *et al*., 1999; Broholm *et al*., 2009). Of the four B class gene candidates, (*GDEF1*, *GDEF2*, *GDEF3,* and *GGLO1*) both *GGLO1* and *GDEF1* were found to be expressed exclusively in floral tissues, whereas *GDEF2* and *GDEF3* transcripts were also detected, although only weakly, in vegetative leaves and petioles. *GGLO1*, *GDEF2*, and *GDEF3* were strongly expressed in petals and stamens. However, *GDEF2* and *GDEF3* gave a weak signal in pappus bristles (whorl 1) and carpel (Yu *et al*., 1999; Broholm *et al*., 2009). The *Gerbera* C class MADS box genes *GAGA1* and *GAGA2* is expressed in the central part of each flower primordium. Later, they were expressed, beginning at early stages of development and continuing throughout, in whorls 3 and 4 (Yu *et al*., 1999). This kind of expression pattern closely resembles the C class genes of *Arabidopsis* and *Antirrhinum* namely *AG* and *PLE* (Yanofsky *et al*., 1990; Davies *et al*., 1999).

1.3 PPIs of *Gerbera* MADS-box genes

There are several genes found in *Gerbera* that are similar to the *Arabidopsis* A class MADS-box gene *AP1* and its paralogs, *CAL* and *FUL*, as well as the orthologue in *Antirrhinum*, *SQUA*. Using GAL4 Y2H assays, it was observed that the GGLO1 protein can form a heterodimer with all the other three *Gerbera* B class MADS-box domain proteins namely GDEF1, GDEF2, and GDEF3 (Broholm *et al*., 2009; Ruokolainen *et al*., unpublished results). Floral homeotic C function genes *GAGA1* and *GAGA2* form active heterodimers with the SEP-like *Gerbera* E class

proteins GRCD1, GRCD2, and GRCD5 in case of pair-wise interaction assay but they do not interact between themselves (Yu *et al.*, 1999; Teeri *et al.*, 2006).

2. Background and aims of this study

The complex regulation of diverse biological processes acting in eukaryotic organisms is only possible through interactions between different components in the cell. Many methods exist to detect protein-protein interactions in plant cells, but these methods come with many limitations, hence, more efficient methods need to be introduced and developed to achieve better results with fewer drawbacks. The general aim of this project was to examine the practicability of identifying PPIs *in planta* with split *Renilla* luciferase in order to expand the list of methods currently used to understand protein-protein interactions *in planta*.

Inflorescence development in the large sunflower family Asteraceae (e.g., *Gerbera hybrida*) has distinct features which are absent in the traditional model plant systems. Plant MADS-box genes act as homeotic selector genes in flower development and induction. B class MADS-box genes are responsible for specifying floral petal and stamen identities, so PPI analysis is necessary to identify specific roles and activities of B class MADS-box genes. The traditional Y2H/Y3H, BRET, FRET, and BiFC methods are able to answer the question of which *Gerbera* MADS-box domain proteins are capable of interaction when brought in close proximity of each other in cellular context, but these methods have a lot of limitations. For example, the Y2H/Y3H system has a tendency to produce false positives as well as false negatives by showing reporter gene activity where no protein-protein interaction is involved. False negatives involve physiological protein-protein interactions that are not detected by this particular assay. BRET is disadvantaged by a very faint light signal, which makes it difficult to detect energy transfers in animal and plant tissues. FRET and BiFC are technologically challenging for testing a large number of protein pairs. In addition, generation of autofluorescence by the cell wall, chloroplasts, and other cell structures has made the use of FRET and BiFC assays limited in plant cells (Chen *et al*., 2008). Lastly, photobleaching of the donor fluorophore and phototoxicity caused by the external light source are also two major drawbacks which have ultimately restricted their use *in planta* (Xu *et al*., 2007; Held *et al*., 2008). Thus, a potentially more quantitative method (i.e., split *Renilla* luciferase) was employed to detect the PPIs of interesting *Gerbera* B class MADS-box genes (**Figure 5**).

The specific aims of the research work were:

(1) To test the effectiveness of PPIs with split *Renilla* luciferase in plant system.

(2) To identify PPIs among three well known *Gerbera* B class MADS-box domain proteins (GDEF1, GDEF2, and GGLO1).

(3) To compare the effectiveness of electroporation and polyethylene glycol (PEG) mediated transformations *in planta*.

3. Materials and methods

3.1 Vector construction

3.1.1 Collection of entry vectors

3.1.1.1 Features of entry vectors

For conducting this experiment, a total of 5 entry vectors were used (**Table 2**, **Figure 3**). *Arabodopsis* cDNA clones Histone 2A (H2A) and Histone 2B (H2B) were obtained from Naohiro Kato (Louisiana State University, USA). *Gerbera hybrida* cDNA clones DEFICIENS LIKE-1 (GDEF1), DEFICIENS LIKE-2 (GDEF2), and GLOBOSA LIKE-1 (GGLO1) were obtained from Satu Ruokolainen (University of Helsinki, Finland).

Table 2: Entry vectors used and their features.

Name	Gateway® site	Coding gene	Presence of stop codon	Selection
pENTR-H2A	attL1 and attL2	*Arabidopsis* Histone 2A	No	Zeomycin
pENTR-H2B	attL1 and attL2	*Arabidopsis* Histone 2B	No	Zeomycin
pENTR-GDEF1	attL1 and attL2	*Gerbera* DEFICIENS LIKE-1	Yes	Kanamycin
pENTR-GDEF2	attL1 and attL2	*Gerbera* DEFICIENS LIKE-2	Yes	Kanamycin
pENTR-GGLO1	attL1 and attL2	*Gerbera* GLOBOSA LIKE-1	Yes	Kanamycin

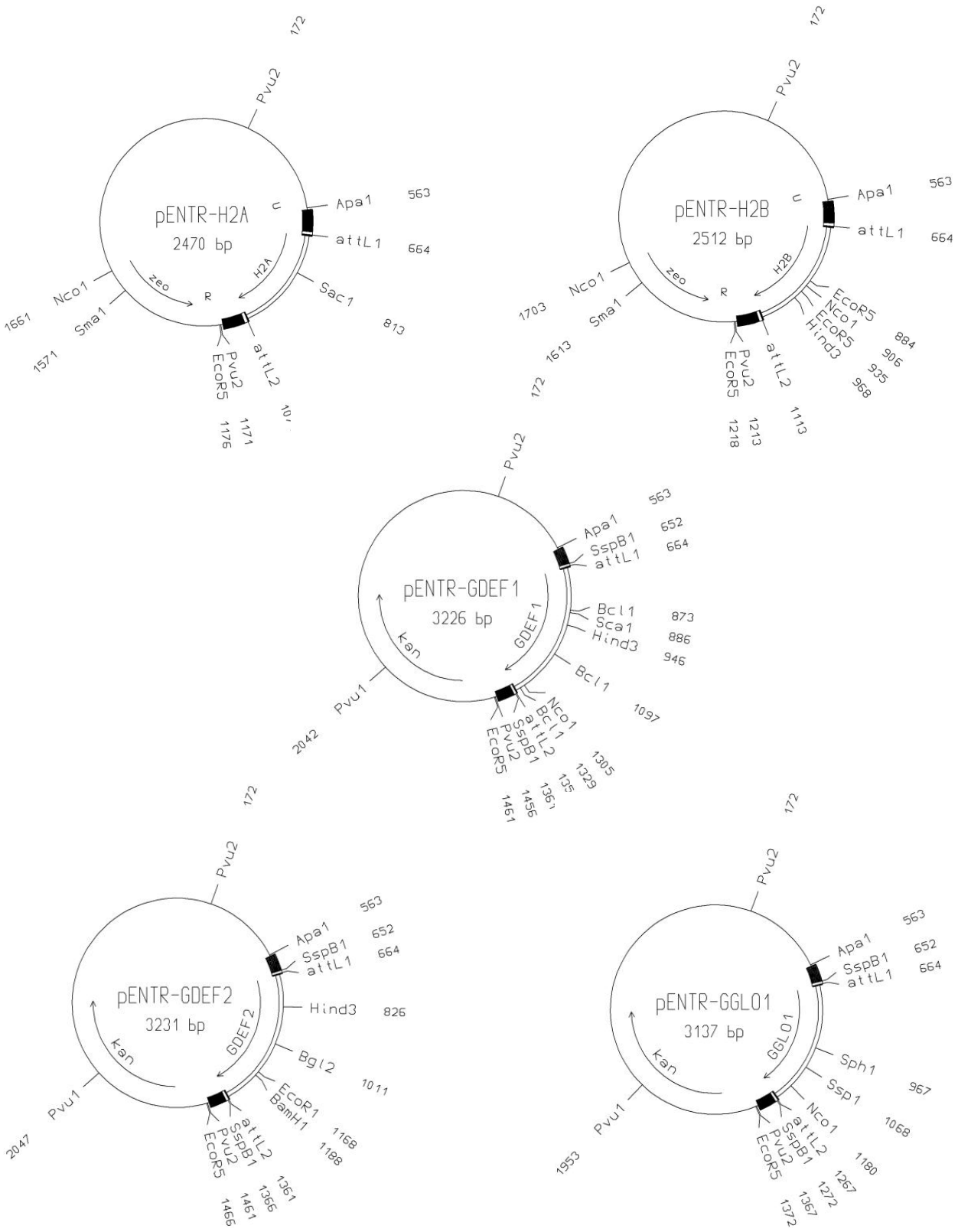

Figure 3: Plasmid map of pENTR-H2A, pENTR-H2B, pENTR-GDEF1, pENTR-GDEF2, and pENTR-GGLO1 entry vectors.

3.1.2 Collection of destination vectors

3.1.2.1 Features of destination vectors

All the 3 destination vectors (pDuExP, pDuExB, and pDuExB2) were provided by Naohiro Kato (Louisiana State University, USA). pDuExP vector encodes the Gateway® recipient site and the Cre-loxP recipient site. This vector also has the N-terminal fragment of *Renilla* luciferase protein [amino acids (aa) 1 – 229, NRLuc] on the 5´-end of the Gateway® recipient site and the 6xHis peptide motif. pDuExB vector encodes the Gateway® recipient site and the Cre-loxP donor cassette. It also encodes the C-terminus fragment of the *Renilla* luciferase protein [(aa) 230 – 311, CRLuc] on the 3´-end of the Gateway® recipient site and the TetraCys peptide motif. pDuExB2 vector encodes the Gateway® recipient site and the Cre-loxP donor cassette. It also encodes the C-terminus fragment of the *Renilla* luciferase protein (CRLuc, 81 aa) on both 5´ and 3´ ends of the Gateway® recipient site and the TetraCys peptide motif (**Table 3**, **Figure 4**).

Mentioned that Cre-loxP is a special type of site-specific recombination system where Cre (a 38 kDa recombinase protein) mediates intra and intermolecular site specific recombination between loxP sites. 6xHis tags are often used for affinity purification of genetically modified proteins. TetraCys tags can bind with the FlAsH (a fluorescein derivative) reagent and become highly fluorescent in green making it possible to detect protein localization. These special features were not used for any purposes in conducting this experiment.

Table 3: Destination vectors used and their features.

Name	Gateway® site	Tag	Plant promoter	Selection
pDuExP	attR1 and attR2	6xHis-NRLuc	35S	Ampicillin
pDuExB	attR1 and attR2	CRLuc-TetraCys	35S	Ampicillin
pDuExB2	attR1 and attR2	CRLuc and CRLuc-TetraCys	35S	Ampicillin

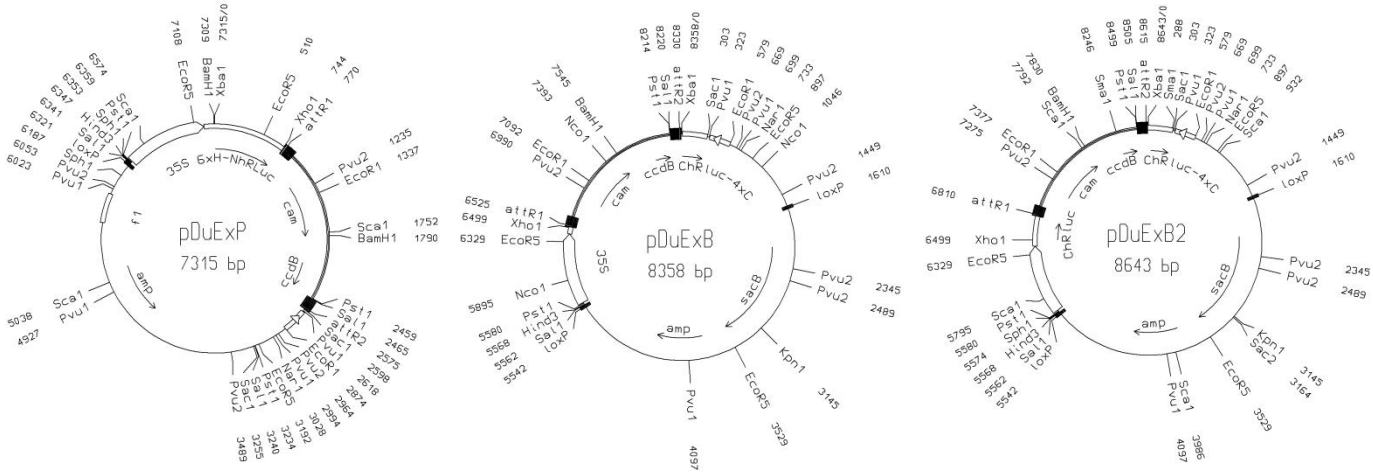

Figure 4: Plasmid map of pDuExP, pDuExB, and pDuExB2 destination vectors.

3.1.3 Construction of recombinant plasmids by Gateway® cloning

In a 1.5 ml Eppendorf tube at RT, 150 ng of entry vector and 150 ng of destination vector were combined in 10 µl TE buffer (pH 8.0). All were mixed well by vortexing briefly. In this way a total of 12 tubes were prepared with different entry and destination vector combinations (**Table 4**, **Figure 7**). The LR clonase® II enzyme mix was thawed on ice for about 2 minutes and vortexed. To each sample, 2 µl of LR clonase® II enzyme mix was added and mixed well by vortexing briefly twice. The reactions were incubated at + 25°C for 1 hour. 1 µl of the Proteinase K solution was added to each sample to terminate the reaction. Finally, samples were incubated at + 37°C for 10 minutes.

3.2 Preparation of competent *E. coli* DH5α cells

A single *E. coli* DH5α colony was inoculated in 5 ml of LB media. They were then grown over night at + 37^{o} C with shaking. 0.5 ml of saturated culture was then sub-cultured into 50 ml of LB media in a 1 liter sterile Erlenmeyer flask and grown at + 37^{o} C for two and a half hours or until OD_{600} was 0.50. Bacterial cells were then chilled on ice for 0.5 – 2 hours in a 50 ml Falcon tube in a cold room at + 4^{o} C. Cells were spun at 2000×g (4000 RPM) for 5 minutes at + 4^{o} C. The pellet was then resuspended in 15 ml of cold 100 mM $CaCl_2$ and chilled on ice for 20 minutes. Centrifugation was repeated and pellet was resuspended in 1 ml of cold 100 mM $CaCl_2$. Finally, cells were chilled on ice for 30 minutes to 24 hours before transformation.

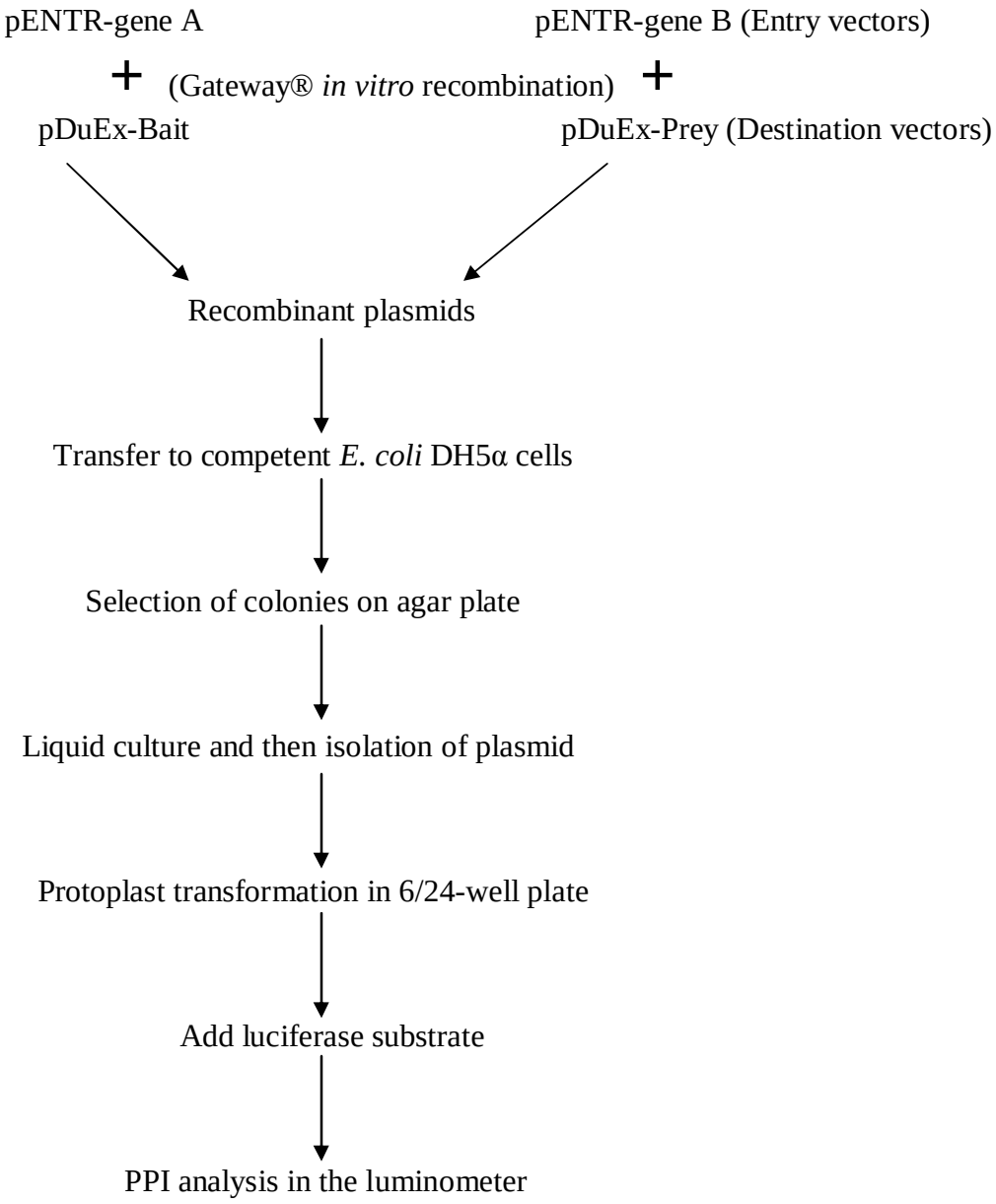

Figure 5: Flow chart of whole system overview.

A pair of cDNAs of interest inserted in the gene expression vectors pDuEx-Bait and pDuEx-Prey were mixed and used to transiently transform plant protoplasts in a 6/24-well plate with either electroporation or PEG. PPIs were detected based on *Renilla* luciferase activities gained by complementation of the split luciferase proteins that translationally fused to the bait and prey proteins respectively.

3.3 Transformation of competent cells with recombinant plasmids

Previously prepared *E. coli* DH5α competent cells were taken from ice and 4 µl recombinant reaction mixture was added to the competent cells and mixed well. After 30 minutes on ice, the cells were placed in a + 42^{o}C incubator for 30 seconds followed by the addition of about 1 ml

LB media to each tube. They were then kept at RT for 30 minutes. *E. coli* DH5α cells were then spun down (with 2500 RCF for 3 minutes at + 4^{o}C) by centrifugation, and the supernatant was discarded. Subsequently, the cells were mixed in the remaining liquid by vortexing. Finally, the cells from each tube were pipetted into individual Petri dishes containing grown media and the selection antibiotic (ampicillin) and were then spread out with a flamed glass triangle. The plates incubated at + 37^{o}C for overnight.

3.4 Culture of cells bearing recombinant plasmids

All bacterial cells were grown in 12 separate Petri dishes overnight at + 37^{o}C with ampicillin as a marker. After the Gateway® reaction, only the *E. coli* DH5α cells bearing 12 entry clones survived because they all had ampicillin genes. Additionally, two extra *E. coli* DH5α cells were grown in two separate Petri dishes containing pANU6, bearing full length firefly luciferase (FLuc), and pHTT672, bearing full length *Renilla* luciferase (RLuc) genes. These two were taken from Gerbera laboratory plasmid collections (**Figure 6**).

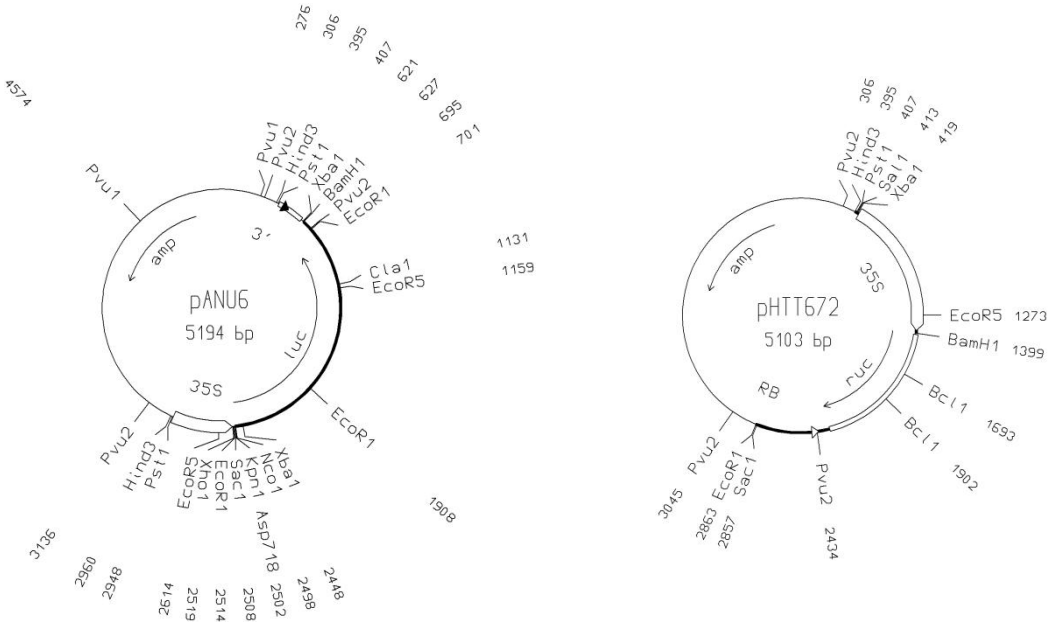

Figure 6: Plasmid map of pANU6, and pHTT672 bearing full length *Renilla* and firefly luciferase, respectively.

Table 4: Newly constructed recombinant plasmids with their expressing genes. Also showing their origin vectors (entry and destination) constructed by Gateway® cloning technique.

Name of the recombinant plasmids	Expressing gene	Entry vector	Destination vector
pSOT1	NRLuc-H2A	pENTR-H2A	pDuExP
pSOT2	NRLuc-H2B	pENTR-H2B	pDuExP
pSOT3	NRLuc-GDEF1	pENTR-GDEF1	pDuExP
pSOT4	NRLuc-GDEF2	pENTR-GDEF2	pDuExP
pSOT5	NRLuc-GGLO1	pENTR-GGLO1	pDuExP
pSOT6	H2A-CRLuc	pENTR-H2A	pDuExB
pSOT7	H2B-CRLuc	pENTR-H2B	pDuExB
pSOT11	CRLuc-H2A-CRLuc	pENTR-H2A	pDuExB2
pSOT12	CRLuc-H2B-CRLuc	pENTR-H2B	pDuExB2
pSOT13	CRLuc-GDEF1	pENTR-GDEF1	pDuExB2
pSOT14	CRLuc-GDEF2	pENTR-GDEF2	pDuExB2
pSOT15	CRLuc-GGLO1	pENTR-GGLO1	pDuExB2

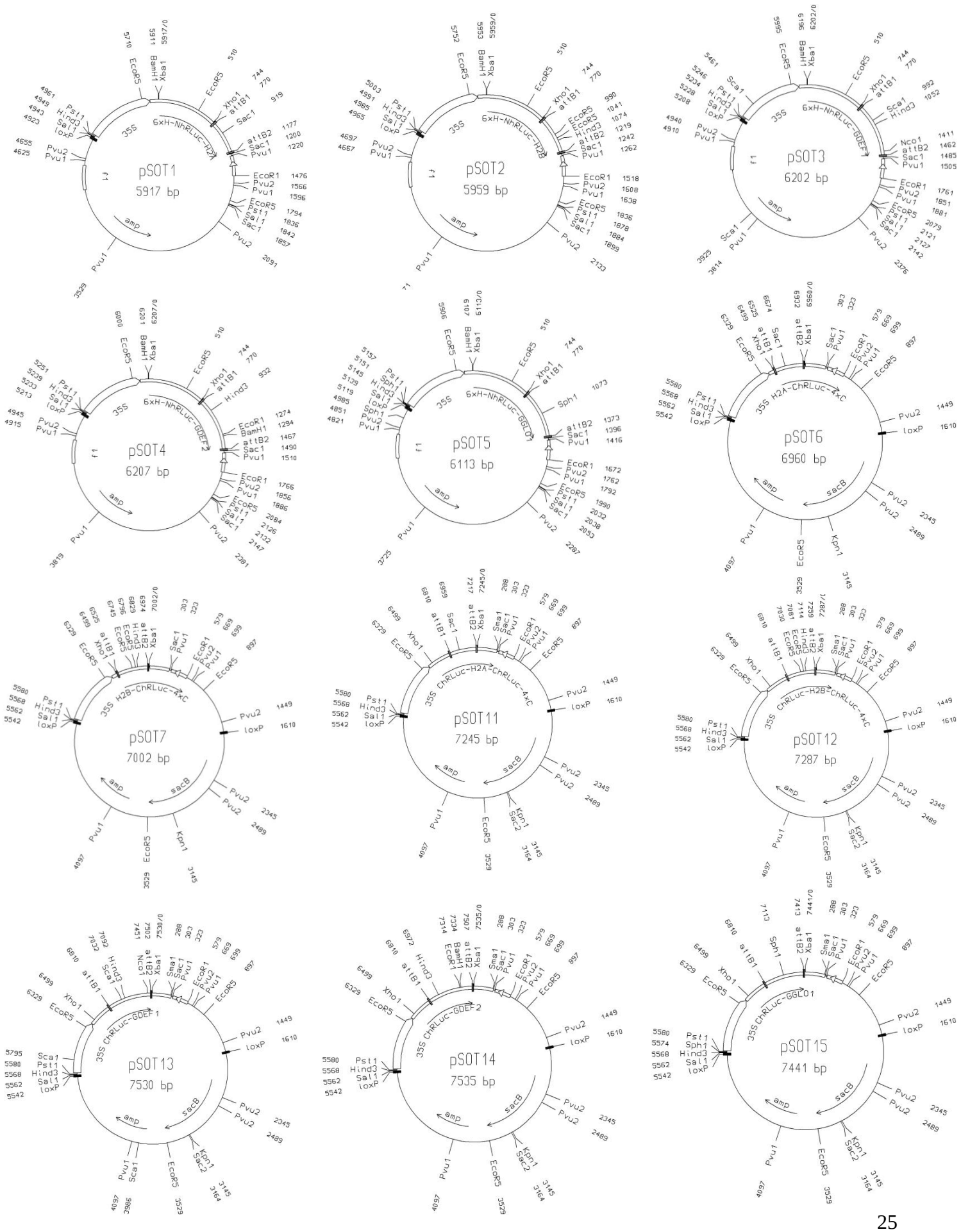

Figure 7: Plasmid maps of 12 vectors constructed in this work by Gateway® cloning of 5 entry and 3 destination vectors.

3.5 Verification of constructed plasmids

3.5.1 Isolation of plasmids by boiling preparation

On each plate more than 10 colonies were formed, but all might not have been the correct insert, so it was then necessary to identify the correct colonies. Plasmids from every colony were isolated and then ran on gel with specific restriction enzymes (based on each plasmid map) in order to identify the correct colonies. Fresh, pure cultures were prepared from each single transformed *E. coli* DH5α cell colony on agar plates with ampicillin for the isolation. 50 µl of STET buffer was then pipetted into each Eppendorf tube and placed on ice. A boiling water bath was prepared simultaneously. A toothpick was used to scrape a fair amount of bacteria from each colony which was then resuspended by vortexing in STET buffer. Agar was avoided, and all were placed on ice. When all tubes were finished, 4 µl of lysozyme (10 mg/ml in water) was added to each tube and then vortexed briefly. The tubes were boiled for 50 seconds and immediately placed on ice. The tubes were then centrifuged for 5 minutes. The sticky pellet was removed from each tube with a toothpick, and 50 µl cold isopropanol was added and mixed well. Tubes were incubated approximately 10 minutes at – 20^{o} C and centrifuged for 10 minutes in + 4^{o} C at 14000 RPM. Supernatant from each tube was removed and 100 µl cold 70% ethanol was added. Tubes were spun for 1 minute in cold (+ 4^{o} C) at maximum speed (14000 RPM). Ethanol was removed and the pellets, which were then dried in vacuum for 5 minutes. Finally, the pellets were resuspended in 20 µl TE buffer.

3.5.2 Digestion of all isolated plasmids with restriction enzymes

After isolation of plasmids from sampled colonies (from each Petri dish) it was necessary to verify the correct inserts. 4 µl of individual plasmid DNA was added along with 9.5 µl DDW and 1.5 µl 10x CA to each 1.5 ml Eppendorf tube. When all were finished, they were treated with restriction enzymes (0.2 µl of each) and incubated for 1 hour at 37°C in an incubator inside a large sand plate. After incubation 3 µl of TD (RNase included) was added.

3.5.3 Identification of desired plasmids by gel electrophoresis

First the comb of the electrophoresis apparatus was ensured to be in its proper place. Hot agarose (0.8% agarose in 1x TBE buffer melted in microwave oven) was poured into the gel space until it

reached the top of the gel box. Appropriate amount of ethidium bromide (depending upon gel size) was added to visualize DNA bands under UV light. It took around half an hour to harden the gel. When the agarose gel had hardened, the stoppers were taken out and TBE buffer solution was poured over the gel, covering it completely with some excess. The comb was gently removed and a pipette was used to load each dye into a separate well in the gel. Previously prepared Lambda DNA digested with *Pst*I was always used as a ladder. Then all prepared digested plasmids were loaded in each well. A separate pipette tip was used for each sample. A diagram was drawn on a sheet of paper to keep track of samples loaded in particular wells. Finally, the gel was run (time was a variable depending upon the size of the gel) and the results were scanned for analysis. In this way, the 12 needed bacterial clones were selected for this experiment. The selected 12 colonies were pure cultured in separate Petri dishes for further use and conservation.

3.6 Large-scale isolation of plasmids by Maxi Kit

From the Petri dishes the needed bacterial colonies (pSOT1, pSOT2, pSOT3, pSOT4, pSOT5, pSOT6, pSOT7, pSOT11, pSOT12, pSOT13, pSOT14, and pSOT15) were liquid cultured overnight in 250 ml flask with selection antibiotic (ampicillin) at + 37^{o} C. After overnight culture, plasmids were isolated by Maxi Kit (Qiagen®) from those liquid cultured bacterial cells. All the steps were done according to the manufacturer's protocol.

3.7 Plant material and their growth conditions

Nicotiana tabacum cv. SR1 was grown in pots containing soil and organic fertilizer in a growth chamber at + 25^{o}C with ambient humidity. Normally, plants of four weeks or older were used in this experiment.

3.8 Protoplast preparation

Five pieces of 10 – 20 cm long tobacco leaves were taken and cut into two or more pieces that allowed them to fit into a 1000 ml glass beaker. Under laminar air flow, the leaf pieces were surface sterilized in 70% ethanol for 1 minute, then rinsed in sterile distilled water three times. They were then submerged in commercial NaOCl solution (dilution 1: 3) for 10 minutes. Finally, the leaves were washed five times with sterile water to remove NaOCl. In a small amount of

Man-pp (1x) on a Petri dish, the damaged outer rims of leaf pieces were trimmed off. The leaf pieces were cut into 1 mm thin sections with a sharp surgical blade, and the mid vain area was avoided. The cut material was collected in a 9 cm Petri dish on the surface of 20 ml Man-pp (1x) and plasmolysed for 30 minutes. The whole surface area of the Petri dish was filled with 1 mm thin leaf sections. The tissue was always kept upside down (lower epidermis up) so that the stomata would always be unblocked. The medium was replaced with 10 ml of filter sterilized Enzyme solution. The plate was covered lightly with aluminum foil and kept in the laminar hood for 16 hours. After 16 hours the material was disturbed by swinging it gently. The tissue was more or less completely digested. The suspension was pipetted gently through a 100 μm nylon net into a 50 ml Falcon tube. It was spun for 10 minutes at 500 RPM and + 22^{o}C. The supernatant was removed, and the pellet was gently resuspended in three ml of 50% Percoll (Sigma) in Man-pp (2× Man-pp was used to prepare the Percoll solution). 8 ml of 20% Percoll was pipetted in a sterile 15 ml Falcon tube. Then the protoplasts were carefully pipetted in 50% Percoll below to the 20% Percoll. Finally, 1 ml of Man-pp (1x) was added on top of the 20% Percoll followed by spinning for 10 minutes at 1000 RPM and + 22^{o}C. The protoplasts were carefully pipetted on top of the 20% Percoll into a new weighted 15 ml Falcon tube. Excess of the Man-pp medium was avoided.

3.9 Transformation of protoplasts with selected plasmids

3.9.1 PEG mediated transformation

After isolation of the protoplasts, the next step was to transform them with constructed plasmid vectors for conducting PPI analysis. PEG mediated transformation, the protoplasts were transferred into a weighed 15 ml Falcon tube, and the tube was filled with MMg solution. The tube was spun for 10 minutes at 500 RPM and + 22^{o}C, and the supernatant was removed. The tube was weighed again, and the amount of protoplasts was calculated. 10 mg (about 0.25 Million) cells were used per standard experiment (total volume 100 μl). Again, the protoplasts were washed with MMg solution (10 mg cells/100 μl). 10 μl DNA (eqv. to 10 μg plasmid) was added to a 2 ml round bottom Eppendorf tube, then 100 μl of the protoplasts was added and mixed gently. Immediately, 100 μl PEG solution was added, and mixed gently. Cells with DNA were incubated for five to 30 minutes at room temperature. After incubation, 0.4 ml W5 solution

was added and mixed gently. Then the mixture was spun at 100×g (1030 RPM) for 2 minutes, and the supernatant was removed. The protoplasts were resuspended in 100 µl WI solution and transferred to 24-well plate with additional 0.5 ml of WI solution. Finally, plates were covered with paper and kept at RT for 16 hours. After 16 hours, the plates were gently rocked to loosen the cells from the bottom. With a cut tip, cells were collected into a 1.5 ml Eppendorf tube and analyzed for luciferase activity.

3.9.2 Electroporation

Due to non-consistent results of PEG mediated transformation, later electroporation was carried out for analyzing PPIs. The new, weighed 15 ml Falcon tube containing protoplasts was filled with Aa buffer. It was spun for 10 minutes at 500 RPM at + 22^{o}C. The supernatant was removed. The tube was then weighed, and the amount of protoplasts was calculated. 0.25 Million (about 10 mg) cells were used per standard experiment (total volume 200 µl). Then the protoplasts were washed into Aa buffer (10 mg cells/200 µl). 200 µl pp-Aa buffer solution was transferred into one electroporation cuvette. 10 µl DNA (eqv. to 10 µg plasmid) was added to the electroporation cuvette and mixed gently by tapping the cuvette. The cuvettes were allowed to cool on ice for 10 minutes. The cells were mixed gently, and an electric pulse (defended by a combination of Voltage and Capacitance, e.g., 200 V and 750 µF) was given. Using a new Gene Pulser Xcell™ Electroporation System (Bio-Rad) the experimental electroporation method was employed. According to the manufacturer's manual, this machine is optimized for electroporation of most eukaryotic cells including mammalian cells and plant protoplasts. After electric pulse, the protoplasts were left on ice for a further 10 minutes. Then, the cells were washed with K3-man-MES with hormones solutions from the cuvettes into a 6-well plate. Next they were covered with a paper hand towel and incubated at RT for 16 hours. After 16 hours, 0.8 – 1 ml of the clear supernatant was carefully removed from each plate. Then the plates were gently rocked to loosen the cells from the bottom. With a cut tip, they were collected into a 1.5 ml Eppendorf tube and measured for luciferase activity.

3.10 Quantitative assay with the Dual-luciferase® kit

The Dual-Luciferase® Reporter Assay System provides an efficient means of performing two reporter assays. The activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*)

luciferases were measured sequentially from a single sample. The firefly luciferase reporter was measured first by adding Luciferase Assay Reagent (LARII) to generate a luminescent signal lasting at least 1 minute. After quantifying the firefly luminescence, this reaction was quenched, and the *Renilla* luciferase reaction was initiated simultaneously by adding Stop & Glo® Reagent to the same sample.

Dual-luciferase® substrate was taken from the – 70° C freezer, melted at RT in a water bath, and mixed well before pipetting 50 µl into the luminometer cuvettes. Stop & Glo® Substrate and Stop & Glo® Buffer were taken from the – 20° C freezer and were similarly melted at RT, and 20 µl of 50x Stop & Glo® Substrate was added to 1 ml of Stop & Glo® Buffer. The 16 hour incubated PEG transformed 24-well plate protoplasts were taken and collected into individually marked 1.5 ml Eppendorf tubes. All were spun at 100×g (1030 RPM) for 2 minutes at RT, and the supernatant was removed. They were then placed on ice. After that, 100 µl ice cold Modified Lux Buffer was added to each Eppendorf tube, and the cells were homogenized with an ice cold pestle. Next the homogenates were spun in a microcentrifuge for 10 minutes at maximum speed (at + 4° C). 10 µl of plant extract was then added to each cuvette containing LARII and quickly mixed by shaking. Each cuvette was then placed into the luminometer chamber. Measurement of firefly activity was accomplished by the instrument after pressing the 'START' button. After measuring firefly activity, 50 µl Stop & Glo® Reagent was added to each cuvette and then quickly mixed by vortexing. The cuvette was again placed into the luminometer chamber and *Renilla* activity was measured by pressing the 'START' button and reading the luminescence value.

4. Results

4.1 Vector constructs

4.1.1 Vector construction by Gateway® recombination

In vitro recombination of pDuEx destination vectors (P, B, and B2) and pENTR entry vectors (H2A, H2B, GDEF1, GDEF2, and GGLO1) were conducted using a Gateway® LR clonase® II enzyme mixture according to the manufacturers protocol (Invitrogen). Normally there should have been 15 reactions because 3 destination and 5 vectors were present, but due to experimental necessity only 12 reactions were conducted. Because 3 entry vectors (GDEF1, GDEF2, and GGLO1) had retained stop codons, they were not recombined with pDuExB. Recombination with pDuExB2 yielded fusions of the *Renilla* luciferase C-terminus to the amino termini of the proteins.

4.1.2 Selection of restriction enzymes for gel electrophoresis

Primarily all 12 of the constructed plasmids and their 3 destination vectors were tested with *Eco*RI, *Hin*dIII, and *Bam*HI restriction enzymes (**Table 5**). For detecting the appropriate fragment size, pre-calculated bacteriophage lambda digested with *Pst*I was used as a DNA size marker. After the primary screening, all plasmids were verified by specific digestions with another set of restriction enzymes. In all cases all constructed plasmids were digested with specific restriction enzyme(s), and the same digestion was always carried out with their originating plasmids (means pDuExP, pDuExB, and pDuExB2 destination vectors), thus acting as a positive control. For example: pSOT1 was digested with *Sac*I + *Eco*RV, and the same digestion was carried out with its originating vector pDuExP (**Table 6**, **Figure 9**).

Table 5: Digestion of 12 recombinant and 3 destination plasmids with *Eco*RI + *Hin*dIII + *Bam*HI, and the calculated fragment lengths.

Plasmid name	**Fragments** (base pairs)	**From**	**Total plasmid length** (base pairs)
pDuExP	3473 1343 1084 962 453	2874 to 6347 7309 to 1337 1790 to 2874 6347 to 7309 1337 to 1790	7315
pSOT1	3473 1482 962	1476 to 4949 5911 to 1476 4949 to 5911	5917
pSOT2	3473 1080 962 444	4991 to 1518 1074 to 5953 5953 to 4991 1518 to 1074	5959
pSOT3	3473 1058 962 709	1761 to 5234 6196 to 1052 5234 to 6196 1052 to 1761	6202
pSOT4	3473 962 938 472 342 20	1766 to 5239 5239 to 6201 6201 to 932 1294 to 1766 932 to 1274 1274 to 1294	6207
pSOT5	3473 1678 962	1672 to 5145 6107 to 1672 5145 to 6107	6113
pDuExB	4989 1524 1392 453	579 to 5568 5568 to 7092 7545 to 579 7092 to 7545	8358
pSOT6	4989 1971	579 to 5568 5568 to 579	6960
pSOT7	4989 1261 752	579 to 5568 5568 to 6829 6829 to 579	7002
pDuExB2	4989 1809 1392 453	579 to 5568 5568 to 7377 7830 to 579 7377 to 7830	8643
pSOT11	4989 2256	579 to 5568 5568 to 579	7245
pSOT12	4989 1546 752	579 to 5568 5568 to 7114 7114 to 579	7287
pSOT13	4989 1524 1017	579 to 5568 5568 to 7092 7092 to 579	7530
pSOT14	4989 1404 780 342 20	579 to 5568 5568 to 6972 7334 to 579 6972 to 7314 7314 to 7334	7535
pSOT15	4989 2452	579 to 5568 5568 to 579	7441

4.1.3 Detection of correct inserts by gel electrophoresis

For every recombination reaction (by Gateway® cloning), there were at least twenty colonies formed with the possibility that all might not be the correct insert. Hence, for every recombination reaction (total 12), at least three colonies were checked, and if all were found to be incorrect, then more colonies were considered for verification. Digested plasmids were run in 0.8% agarose gel followed by scanning with an electrophoresis scanner (**Figure 8**).

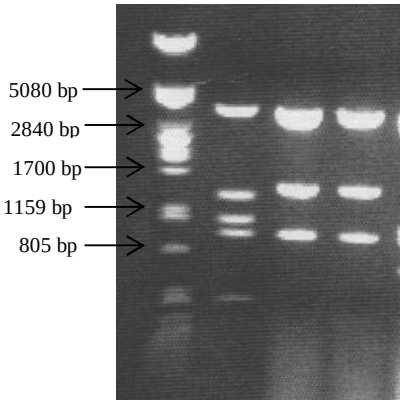

Figure 8: *Eco*RI + *Hin*dIII + *Bam*HI digestion of pSOT1 vector verified with agarose gel electrophoresis. Lanes 1, lambda *Pst*I; 2, pDuExP; 3; pSOT1 (from colony one); and 4, pSOT1 (from colony two) respectively.

After gel electrophoresis verification, both colonies of pSOT1 were found to contain correct inserts, and colony one was arbitrarily selected for future steps. In this manner, all the remaining 11 recombinant plasmids were verified.

Table 6: Digestion of pSOT1 and its originating vector pDuExP with *Sac*I + *Eco*RV and the calculated fragment lengths.

Plasmid name	**Fragments** (base pairs)	From
pDuExP (7315 bp)	3853	3255 to 7108
	2088	510 to 2598
	717	7108 to 510
	594	2598 to 3192
	63	3192 to 3255
pSOT1 (5917 bp)	3853	1857 to 5710
	717	5710 to 510
	594	1200 to 1794
	409	510 to 919
	281	919 to 1200
	63	1794 to 1857

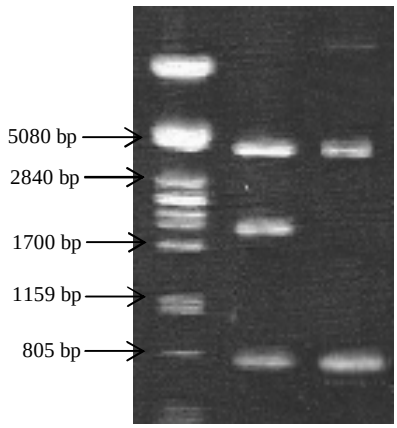

Figure 9: *Sac*I + *Eco*RV digestion of pSOT1 vector verified by agarose gel electrophoresis. Lanes 1, lambda *Pst*I; 2, pDuExP; and 3, pSOT1 (from colony 1) respectively.

After verifying with gel electrophoresis it became clear that colony one of pSOT1 contained the correct insert. In this manner, all 12 recombinant plasmids (pSOT1, pSOT2, pSOT3, pSOT4, pSOT5, pSOT6, pSOT7, pSOT11, pSOT12, pSOT13, pSOT14, and pSOT15) were verified again by specific digestions with restriction enzymes, and in every case only correct constructs were selected for future experiments.

4.2 Use of PEG mediated transformation

4.2.1 Standardization of the amount of protoplasts used for PEG mediated transformation

This was the first time the Gerbera laboratory had tested PEG mediated transformation of plant protoplasts, so it was therefore necessary to determine the number of protoplasts used per transformation and its efficiency. For this investigation, from 0.031 to 0.500 million cells were used to identify the optimum amount of protoplasts for transformation. The PEG treatment was between 5 to 30 minutes. In all experiments 10 µl (5 µl + 5 µl, eqv. to 10 µg) of pANU6 and pHTT672 plasmids containing full length firefly and *Renilla* luciferase, respectively, were used and the luciferase activities were measured 16 hours after the transformation (**Figure 10**).

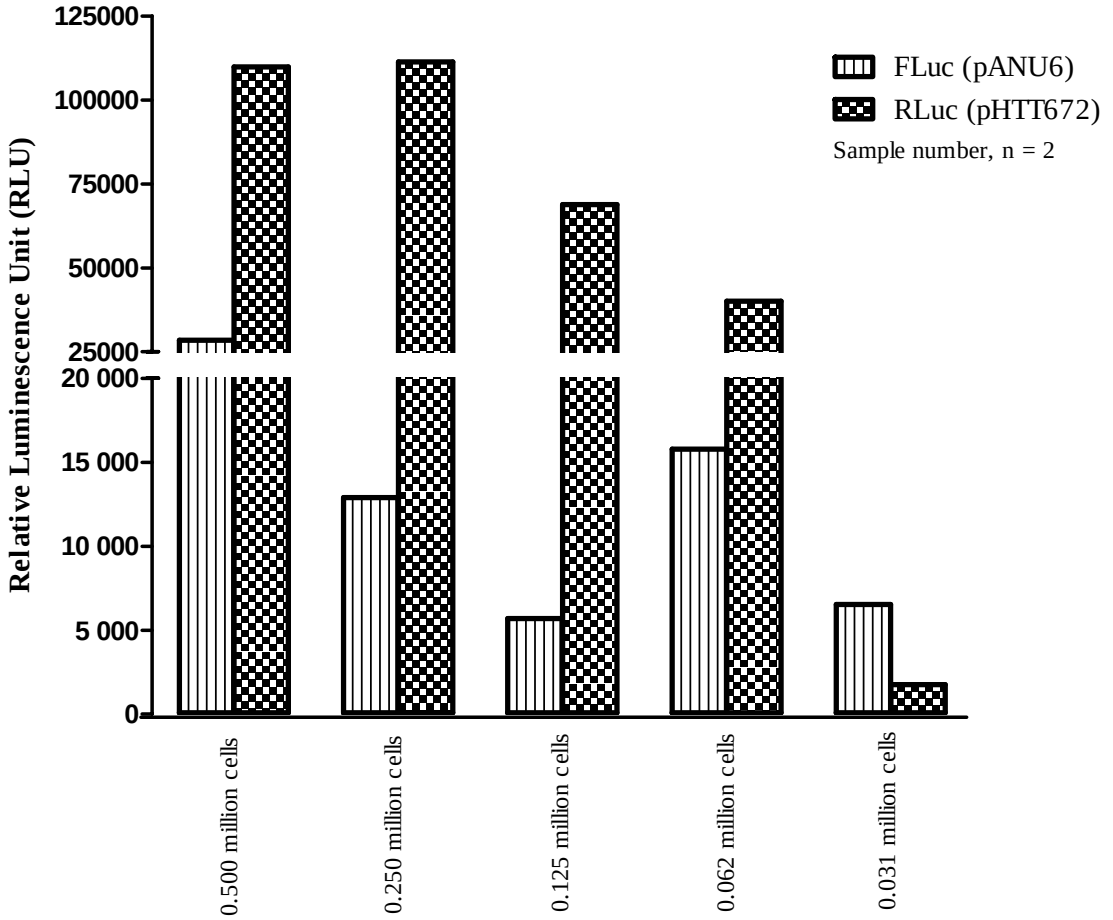

Figure 10: Standardization of amount of protoplasts used for transformation.

From this experiment it was determined that between 0.50 and 0.25 million protoplasts were best suitable for PEG mediated transformation based on higher Relative Luminescence Units (RLUs) in the case of both luciferases. For this investigation, 0.25 million (eqv. to 10 mg) cells per standard experiment (total volume 100 µl) were selected.

The amount of cell injury due to PEG treatment was also observed under the microscope to identify the optimum duration of PEG treatment (**Table 7**, **Figure 11**).

Table 7: Effect of PEG treatment on tobacco protoplasts in different time intervals.

Time duration (Minutes)	**Injured cells** (microscopic observation)
Control	Only occasional
5	Very few
10	Moderate
15	Moderate
20	Moderate
25	Comparatively higher
30	Comparatively higher

However, no considerable differences in cell injury were found with respect to the duration of PEG treatments within 5 to 30 minutes. Therefore, it was concluded that duration does not significantly influence PEG transformation processes.

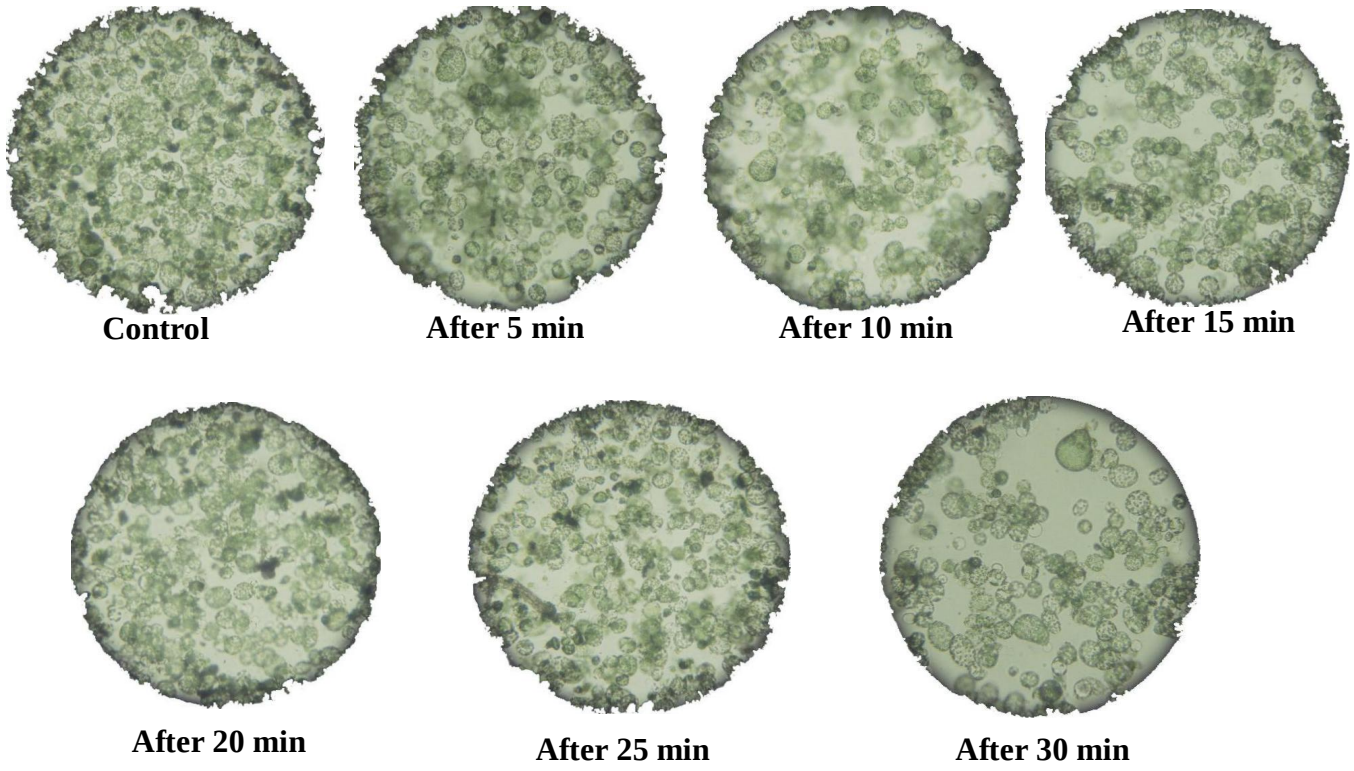

Figure 11: Effect of PEG treatment on protoplasts in different time intervals.

Efficiency of PEG mediated transformation was tested in different time intervals (5, 15, and 30 minutes) and the RLU was measured by a luminometer (**Table 8**). Here, only pANU6 containing firefly luciferase was used as vector.

Table 8: Test of PEG mediated transformation efficiency with firefly luciferase.

Time duration	**Plasmid used**	**Relative RLU** (1st replicate)	**Relative RLU** (2nd replicate)	**Relative RLU** (3rd replicate)
5 min	pANU6	19588	20213	1333501
15 min	pANU6	116647	405	1334
30 min	pANU6	83060	9200	67216

From the above experiment it became obvious that PEG mediated transformation did not give consistent results and transformation efficiency varied a great deal within replicates.

4.2.2 Observation of PPIs with PEG method

First, a well known PPI of *Arabidopsis* H2A and H2B was tested with this method (**Table 9**). Due to its inconsistent behavior with testing vectors (containing N and C terminal domains of *Renilla* luciferase and H2A and H2B genes), another two vectors bearing full length firefly and *Renilla* luciferase were used for clarifying the transformation efficiency. In all experiments protoplasts were transformed with 8 µl (4 µl + 4 µl) of each construct (one Bait and another Prey) and 2 µl (1 µl + 1 µl) of full length firefly (pANU6) and *Renilla* luciferase (pHTT672) in a 24-well plate (total volume 10 µl, eqv. to 10 µg). The luciferase activities were measured 16 hours after the transformation.

Table 9: PEG mediated transformation to identify well studied PPI between *Arabidopsis* H2A and H2B proteins.

Bait	**Prey**	**RLU (*Renilla*)**			**RLU (Firefly)**			**Plasmid name**
-	-	16			13			No plasmid
H2A-CRLuc	NRLuc-H2B	(3 replicates)			(3 replicates)			pSOT6 and pSOT2
		25	22	15	19402	5176	40435	
H2B-CRLuc	NRLuc-H2A	49	18	15	38600	1291	79	pSOT7 and pSOT1
CRLuc-H2A-CRLuc	NRLuc-H2B	22	27	15	4	441	319	pSOT11 and pSOT2
CRLuc-H2B-CRLuc	NRLuc-H2A	18	58	27	302	1789	513	pSOT12 and pSOT1
Full length *Renilla* luciferase		111345						pHTT672
Full length firefly luciferase		28418						pANU6

From these experiments it became clear that PEG mediated transformation was not reliable, and variation among replicates was too high. The PEG method was unable to reliably detect well known PPI between *Arabidopsis* H2A and H2B proteins.

4.3 Use of electroporation

4.3.1 Standardization of electroporation technique

After unsatisfactory results with the PEG method, electroporation method was employed to detect the PPIs among different proteins. First, *Nicotiana tabacum* cv. SR1 protoplasts were electroporated using three different combinations of Voltages (250, 125, and 100 V) and Capacitances (1000, 500, and 250 μF). In this experiment, 0.25 million tobacco protoplasts were transformed with 10 μl ((5 μl + 5 μl, eqv. to 10 μg) of pANU6 and pHTT672 plasmids containing full length firefly and *Renilla* luciferases, respectively, in a 6-well plate and the luciferase activities were measured 16 hours after the transformation (**Figure 12**).

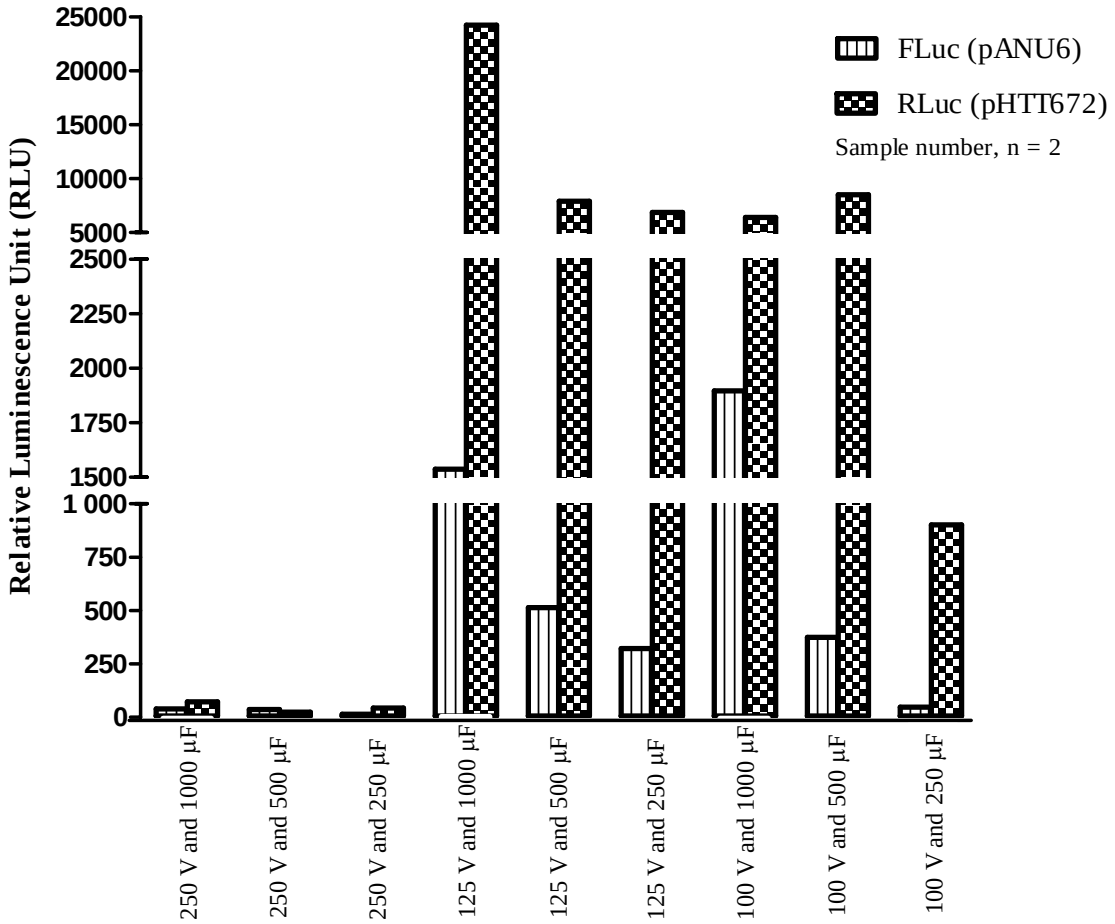

Figure 12: Standardization of electroporation Voltage and Capacitance for tobacco protoplasts transformation.

From the above experiment it was concluded that many combinations (e.g., Voltage 250 V and Capacitance 250 µF) were not suitable for electroporation. Other obtained results were also very much dissatisfactory. For that reason, some new combinations were again set to optimize the method.

Tobacco protoplasts were then electroporated using three different combinations of Voltages (200, 175, and 150 V) and Capacitances (1000, 750, and 500 µF). Here, 0.25 million *Nicotiana* protoplasts were transformed with 10 µl (5 µl + 5 µl, eqv. to 10 µg) of pANU6 and pHTT672 plasmids containing full length firefly and *Renilla* luciferases, respectively, in a 6-well plate, and the luciferase activities were measured 16 hours after the transformation (**Figure 13**).

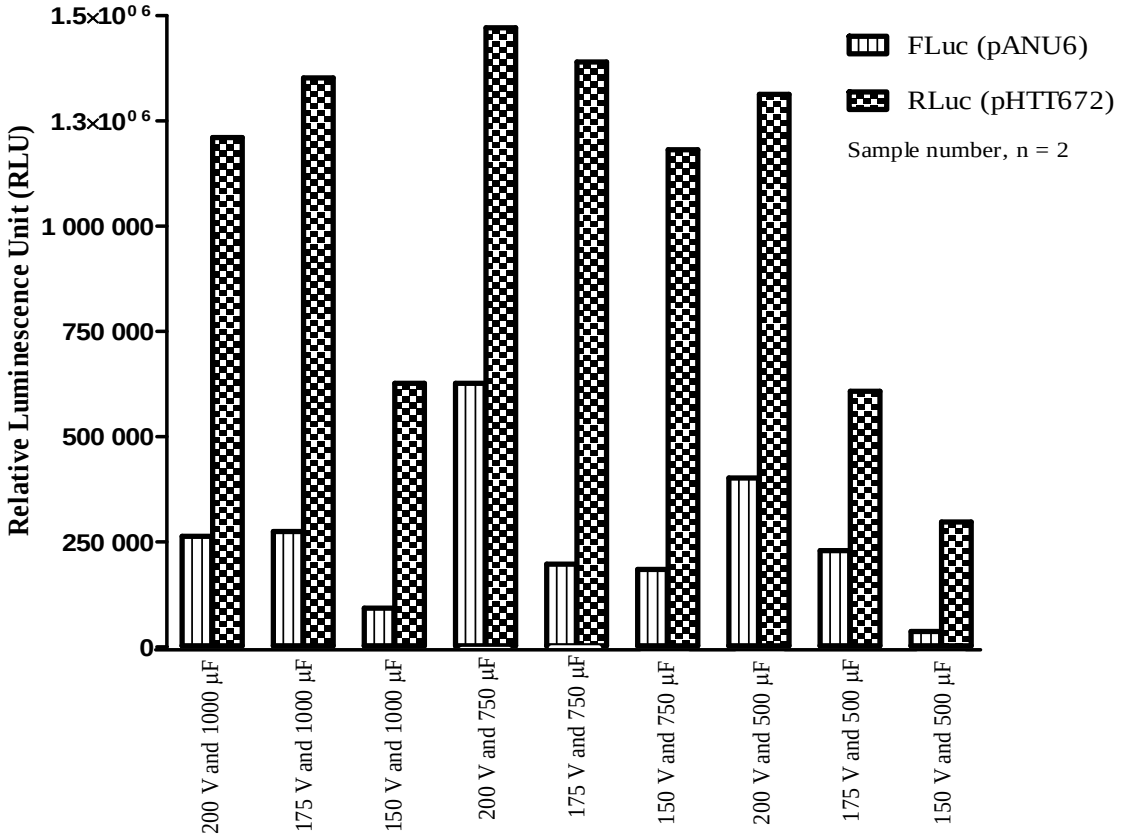

Figure 13: Final optimization of electroporation Voltage and Capacitance for protoplasts transformation.

From this data it was clear that Voltage 200 V and Capacitance 750 µF was the best combination, which gave, on average, the maximum result for both firefly and *Renilla* luciferase assays.

4.3.2 Analysis of PPIs with electroporation

Primarily, the well known PPI of *Arabidopsis* H2A and H2B was tested with this method. As a control, pHTT672, which expresses full length *Renilla* luciferase, was used. 0.25 million tobacco protoplasts were transformed with 8 µl (4 µl + 4 µl, eqv. to 8 µg) of each pDuEx vector and 2 µl of RLuc vector in a 6-well plate. The luciferase activities were measured 16 hours after the transformation. In mock samples, no DNA was added.

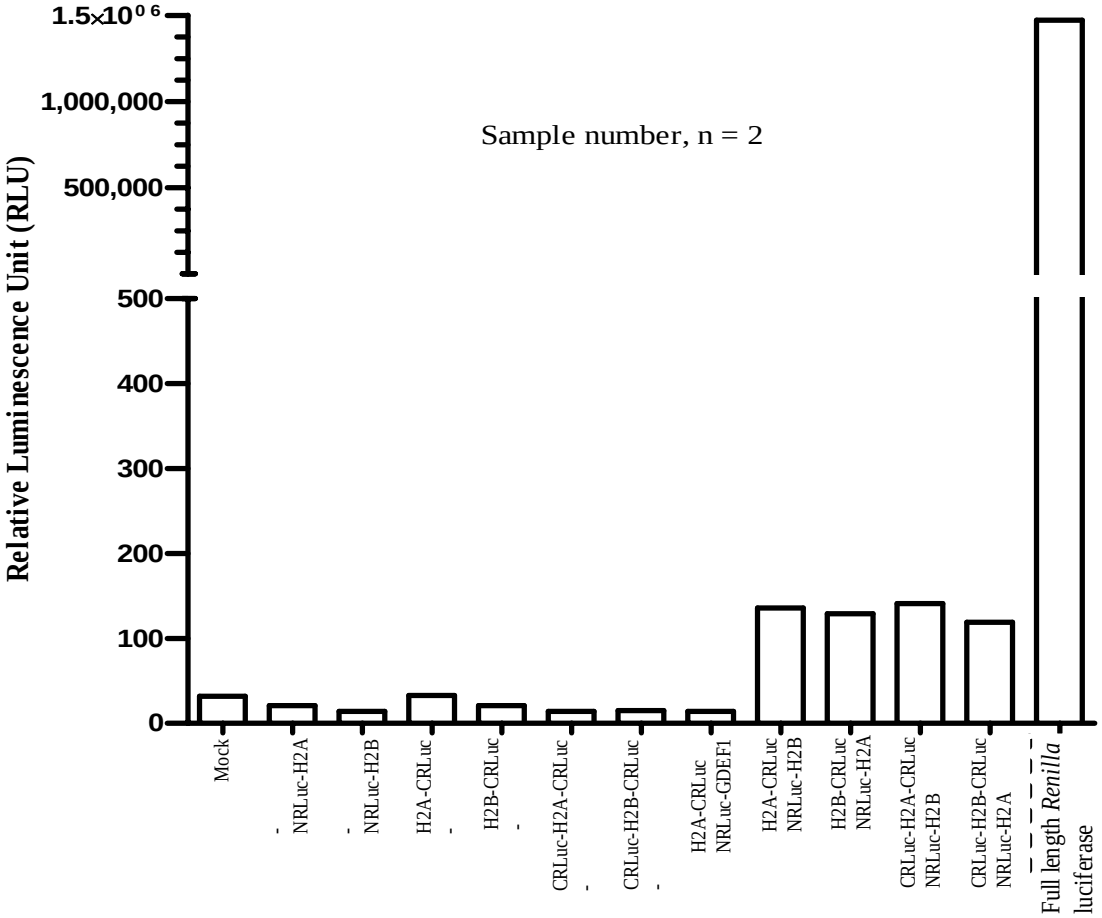

Figure 14: Complemented *Renilla* luciferase activities in *Nicotiana tabacum* cv. SR1 protoplasts expressing *Arabidopsis* H2A and H2B.

The protoplasts transformed with interacting protein pairs pSOT6 + pSOT2, pSOT7 + pSOT1, pSOT11 + pSOT2, and pSOT12 + pSOT1 showed, on average, 9.4-fold higher RLUs than the mock treated protoplasts. In contrast, the protoplasts transformed with the non-interacting plasmid pair (pSOT6 + pSOT3) had only 1.5-fold higher luminescence intensity than the mock-treated protoplasts. It was then calculated that the signal of the H2A-H2B PPI was about 6.3-fold (9.4/1.5) higher than the non-specific signal. Moreover, results from protoplasts transformed with pHTT672 (encodes full length *Renilla* luciferase) and interacting protein pairs (pSOT6 + pSOT2, pSOT7 + pSOT1, pSOT11 + pSOT2, and pSOT12 + pSOT1) showed that split *Renilla*

luciferase had about 0.001% of the luminescence intensity of full length *Renilla* luciferase (**Figure 14**).

Specific PPIs of *Gerbera* B class MADS-box proteins (GDEF1, GDEF2, and GGLO1) were also detected in tobacco protoplasts. 0.25 million protoplasts were transformed with 8 µl (4 µl + 4 µl, eqv. to 8 µg) of each vector and 2 µl of RLuc as a control in a 6-well plate, and the luciferase activities were measured 16 hours after the transformation. In mock samples, no DNA was added.

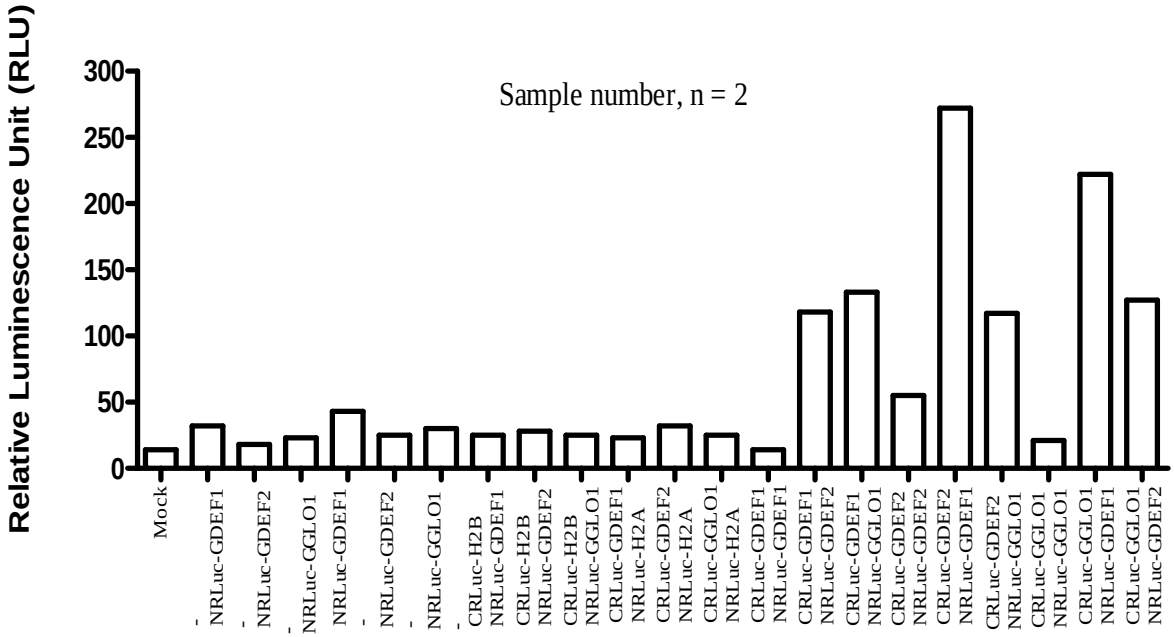

Figure 15: Complemented *Renilla* luciferase activities in *Nicotiana tabacum* cv. SR1 protoplasts expressing GDEF1, GDEF2, and GGLO1 proteins.

The protoplasts transformed with pSOT13 and pSOT4 (expressing CRLuc-GDEF1 and NRLuc-GDEF2) and pSOT7 and pSOT3 (expressing CRLuc-H2B and NRLuc-GDEF1) showed 8.4-fold and 1.8-fold higher luminescence intensity, respectively, than the mock treated protoplasts. GDEF1-GDEF2 had about 4.7-fold (8.4/1.8) greater luminescence intensity than the positive control H2B-GDEF1. From these results it was clear that the split *Renilla* luciferase assay successfully detected specific protein interactions in nuclear proteins (**Figure 15**).

Table 10: Comparison of interacting protein pairs with mock treated samples.

Bait	Prey	RLU	Plasmid pairs	Comparison with mock treated protoplasts
CRLuc-GDEF1	NRLuc-GDEF2	118	pSOT13 and pSOT4	8.4-fold
CRLuc-GDEF2	NRLuc-GDEF1	272	pSOT14 and pSOT3	19.4-fold
CRLuc-GDEF1	NRLuc-GGLO1	133	pSOT13 and pSOT5	9.5-fold
CRLuc-GGLO1	NRLuc-GDEF1	222	pSOT15 and pSOT3	15.8-fold
CRLuc-GDEF2	NRLuc-GGLO1	117	pSOT14 and pSOT5	8.3-fold
CRLuc-GGLO1	NRLuc-GDEF2	127	pSOT15 and pSOT4	9.1-fold

It was found that all three *Gerbera* B class MADS-box domain proteins had PPIs. No homodimer formation was detected, but all heterodimers showed activity. This includes the dimer between GDEF1 (TM6-like MADS domain protein) and GDEF2 (AP3-like protein) not described before (**Table 10**).

5 Discussion

5.1 A brief word about PEG method

PEG mediated transformation is very simple and efficient because it allows simultaneous processing of many samples and yields a transformed cell population with high survival and division rates (Potrykus, 1991; Wang *et al*., 1992; Mathur and Koncz, 1998). Negrutiu *et al*., (1987) first developed this method for direct gene transfer in tobacco protoplasts. This simple method only requires inexpensive supplies and equipment, and it helps in overcoming the range limitations that are involved with *Agrobacterium* mediated transformation. In this experiment, PEG mediated transformation was adapted from He *et al*., (2007).

5.2 A brief introduction about electroporation

Electroporation as a tool for transformation is successfully accomplished in many plant species, for example: tobacco, rice, and wheat (De la Pena *et al*., 1987; Abdul-Baki *et al*., 1990; Zaghmout and Trolinder, 1993). Protoplasts in general are a convenient model to study events that occur rapidly *in planta*, and electroporation has been used extensively over the years for transient and integrative transformation of protoplasts (Fromm *et al*., 1985; Shillito *et al*., 1985). Many laboratories use electroporation for direct gene transfer into tobacco leaf protoplasts to study transient expression (Gallois *et al*., 1995).

5.3 Comparison of electroporation and PEG mediated protoplast transformation

Fujikawa and Kato (2007) successfully transformed *Arabidopsis* protoplasts with excellent efficiency (about 54%) using the PEG method, but this experiment did not work in a consistent manner. Analysis was conducted about the feedback but failed to detect any specific reason behind it. The electroporation method was then employed with a new Gene Pulser Xcell™ Electroporation System (Bio-Rad). According to manufacturer's manual, this machine is optimized for electroporation of most eukaryotic cells including mammalian cells and plant protoplasts. For this experiment, at the time of standardization of optimum Voltage and Capacitance for protoplast electroporation, some challenging data was obtained. For example, with Voltage 250 V and Capacitance 1000 μF (**Table 9**), luciferase did not work at all, but later, with Voltage 200 V and Capacitance 1000 μF (**Table 10**) very good results were achieved.

Within a 50 V difference, these huge variations in results were quite confusing. So, further trials are needed for solid optimization of the exact voltage and capacitance needed for electroporation.

5.4 Low signal-to-background ratios for detecting PPIs *in planta* are improved by using split *Renilla* luciferase assay

The principle of split luciferase method is to detect PPIs as luminescence by using a simple luminometer. Fujikawa and Kato (2007) observed high signal-to-background ratios even with lower emission intensities than those in fluorescence-based assays due to the background reduction by the 'on' or 'off' nature of the signal. In the case of BRET, production of autofluorescence is a major drawback which ultimately reduces the signal-to-background ratio as well as object detectability (Xing *et al*., 2008). The FRET method often produces false positives and because of low signal-to-noise ratios, it might not be possible to identify true interactions by using this technique (Churchman *et al*., 2006). But by using the split *Renilla* luciferase method with the experimental protein pairs the signal-to-background ratio was increased significantly. For example, GDEF1-GDEF2, GDEF1-GGLO1 and GDEF2-GGLO1 protein pairs showed 8.4-19.4, 9.5-15.8, and 8.3-9.1-fold higher signals than the mock treated protoplasts. Among the three pairs of protein-protein interactions, the formation of a heterodimer between GDEF1 and GDEF2 was the strongest, followed by the heterodimer between GDEF1 and GGLO1, and then the heterodimer between GGLO1 and GDEF2 (**Table 13**). These results suggest that duplicated DEF-like *Gerbera* proteins make use of their functions through different strengths of the interactions with other proteins involved in specifying floral development. Although these results were not directly compared with BRET and FRET results, it clearly indicates that the split luciferase method has a higher dynamic range for PPI analysis.

5.5 PPI analysis of *Gerbera* B class MADS-box proteins

MADS-box genes are instrumental in the regulation of floral development, yet the evolution of their functions regarding the control of different floral patterning remains unclear (Drea *et al*., 2007). Tsai *et al*., (2008) identified four DEF- like proteins and one GLO- like protein in *Phalaenopsis equestris*, then tested PPIs with Y2H among themselves and found both homo and heterodimer formation within themselves. In the Y2H assay, GGLO1 formed heterodimer with both GDEF1 and GDEF2, and additionally with GDEF3 in *Gerbera hybrida* (Broholm *et al*.,

2009). But in this experiment, the PPI results demonstrate that three *Gerbera* B class MADS-box proteins (GGLO1, GDEF1, and GDEF2) formed only heterodimers among themselves including the GDEF1 and GDEF2 pair, which had not been seen before. The possibility to detect this new interacting pair (GDEF1 and GDEF2), which was previously never detected by Y2H/Y3H, might be attributable to the higher sensitivity of split *Renilla* luciferase. This outcome suggests that various complexes formed among different combinations of these three B class MADS-box proteins may increase the complexity of their regulatory functions, and thus specify the molecular basis of whorl morphogenesis and combinatorial interactions of floral organ identity genes in *Gerbera*. Evolution in *Gerbera* has also most likely led to a duplication in the ancestral B type genes, resulting in GDEF1 and GDEF2, which could explain why they did not form homodimers.

5.6 Drawbacks of the split luciferase assay

A major limitation of split luciferase assay is the non-identifiability of sub-cellular localization of the interaction which could potentially affect its usefulness (Fujikawa and Kato, 2007). Due to lack of scientific proof, it is also unknown whether this method is sensitive enough to detect PPI with native promoters instead of the 35S ones (Fujikawa and Kato, 2007). Paulmurugan and Gambhir (2003) reported that self-complemented molecules can be formed that could produce reporter signals in the absence of PPIs which might be a drawback of using mutant proteins rather than native ones.

6 Conclusion and future work

To monitor different intracellular protein networks, it is essential to have a multireporting system for use with both intact cells and living plants or animals. Applications of various bioluminescence based reporter genes have been well studied in both prokaryotic and eukaryotic cells and in small living animals for a long time. Hence, it is important to generate different optical split reporter proteins with substrate specificity. Many split-reporter reconstitution methods have already been developed to extend our understanding of many important molecular and cellular functions in living cells. In comparison with these methods, luciferase based complementation assays have more sensitivity and a higher dynamic range due to their optimized substrate. The firefly luciferase gene is the most frequently used luciferase marker gene in eukaryotic cells, but this may change because some cases have shown that cells expressing the *Renilla* luciferase gene achieve much higher levels of light emission than the same cells expressing the firefly luciferase gene. One such example is in the case of yeast *Candida albicans*, whose cells show slight bioluminescence activity when expressing the firefly luciferase gene (Srikantha *et al*., 1995). Some plants are perfect examples of when the *Renilla* luciferase gene has occasional superiority over other luciferase genes used as markers. For example, light emission obtained from electroporated alfalfa protoplasts and transgenic tobacco, tomato, and potato plants is much higher when the *Renilla* luciferase gene is used rather than the bacterial or firefly luciferase genes (Mayerhofer *et al*., 1995). Therefore, for this experiment, *Renilla* luciferase was used as the bioluminescence reporter gene.

In plant systems Y2H/Y3H, BFET, FRET, and BiFC are still widely used for PPI analysis. On the other hand, split luciferase based methods (mainly firefly and *Renilla*) are mostly conserved within mammalian cell experiments, but due to the split *Renilla* luciferase technique's detection sensitivity and the ease of quantification, it is more advantageous than other conventional methods used in plant systems (see **'Background and aims of this study'** section for details). This experiment was conducted as a proof for routine use of this system *in planta*, and was able to detect PPIs successfully between well known interacting pairs of *Arabidopsis* H2A and H2B proteins. Afterward, three *Gerbera* B class MADS-box domain proteins (GDEF1, GDEF2, and GGLO1) interactions were also successfully tested with this method.

Recently, a protoplast two hybrid system has been developed for large-scale PPI analysis *in planta* (Ehlert *et al*., 2006). In order to test this new protoplast two hybrid method, analysis of a protein pair with the new method was compared with an analysis of the same protein pair using the Y2H method. Two different network maps were produced. Even in the case of split *Renilla* luciferase, Fujikawa and Kato (2007) found about 2800 RLU signal with the *Arabidopsis* H2A and H2B protein pair using *Arabidopsis* protoplasts. However in this experiment with the same protein pair a maximum of 141 RLU signals were observed using *Nicotiana tabacum* cv. SR1 protoplasts. The discrepancy between the findings of the new test and those of the established test suggest that the host cell environment may affect protein interactions. Also recently, Remy and Michnick (2006) showed that split *Gausia princips* luciferase emits PPI signals 10-fold higher than split *Renilla* luciferase in mammalian cells. Therefore, further improvements need to be achieved in the dynamic range for plants in the near future.

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9. Appendix

9.1 List of chemicals used and their compositions

10x CA: 70 mM $MgCl_2$, 1000 mM KCl, 1mg/ml BSA, 20 mM 2-Mercaptoethanol, and 200 mM Tris-HCl [pH 7.5].

40% (w/v) PEG solution: to make 10 ml of PEG solution, add 4 g of PEG4000 (Fluka) into 3 ml of MQ H_2O, 2.5 ml of 0.8 M Mannitol, and 1 ml of 1M $CaCl_2$.

Aa buffer: 550 mM Mannitol, 35 mM Aspartic acid monopotassium salt, 35 mM Glutamic acid monopotassium salt, 5 mM Calcium gluconate, and 5mM MES [pH: 7.0].

Adjacent salts: 510 mM $CaCl_2.H_2O$, and 312 mM NH_4NO_3.

Enzyme solution: 1x Man-pp, 0.5% Cellulase (750 mg/150 ml), and 0.2% Macerase (300 mg/150 ml).

K3-man-MES with hormones: 2x Man-pp, 1% adjacent salts, 0.1 µg/ml NAA, and 0.2 µg/ml BAP [pH-5.7].

Man-pp (1x): B5 salts, 500 mM Mannitol, 2% Sucrose, and 0.5% MES [pH: 5.7].

MMg solution: 0.4 M Mannitol, 15 mM $MgCl_2$, and 4 mM MES [pH-5.7].

Modified Lux Buffer: 50 mM Na-phosphate, 4% soluble PVP, 2 mM EDTA, and 20mM DTT [pH: 7.0].

STET buffer: 8% Sucrose, 5% Triton X100, 50 mM EDTA, and 50 mM Tris-HCl [pH 8.0].

TBE buffer: for making 1 liter of 10x TBE buffer add 108 g Tris-base, 55 g Boric acid, 40 ml EDTA, and rest DDW to make the volume 1 liter [pH-8.0].

TE buffer: 10 mM Tris-HCl, and 1 mM EDTA [pH 8.0].

W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5mM KCl, and 2 mM MES [pH-5.7].

WI solution: 0.5 M Mannitol, 20 mM KCl, and 4 mM MES [pH-5.7].

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